TREATMENT OF RUMEN ACIDOSIS WITH α-AMYLASE INHIBITORS

The invention described herein relates to the treatment of rumen acidosis, especially chronic acidosis in ruminants, and related conditions.

Rumen acidosis is a well-documented metabolic disease of ruminants caused by over-consumption of readily fermentable carbohydrates, and problems associated with the condition have been known for many years: see Nordlund et al. 1995, Nagaraja et al. 1998, Owens et al. 1998 and Dirksen 1969. Acidosis can be divided into two forms: acute and chronic. We define acute acidosis as a rumen pH between pH 4.0 and 5.0 with elevated ruminal lactate, and chronic acidosis as a rumen pH between 5.0 and 5.5 with normal levels of lactate of up to 5mM. The literature also refers to subacute acidosis, which has rumen pH values below 5.0 but in some cases is associated with high lactate levels and in others is not. We categorise the former case as mild acute acidosis, and the latter as chronic acidosis.

The main cause of acidosis is the consumption of a diet with a high content of readily fermentable carbohydrate and/or which is low in roughage. Chronic acidosis can occur when animals eat large quantities of readily fermentable diets and may occur at any stage in production, or indeed throughout the time that they are on the high concentrate diets. Acute acidosis can occur when a large increase in the amount of concentrate in the diet takes place, for example after calving or on transfer to the feedlot. However it can also occur following a disruption in normal feed intake patterns such as accidental presentation of excess feed or a fasting period followed by overeating. Reduced rumen pH can also be caused by a decrease in the proportion of crude fibre in the diet. The aetiology of acidosis is therefore based on the absolute intake of excessive quantities of carbohydrate and/or an unfavourable proportion of basic foodstuffs in the ration. The type of grain (high moisture corn is more acidosis-inducing than dry-rolled corn or sorghum) and the type of processing (steam flaked grain is particularly digestible) along with type and amount of roughage is important. Grains such as barley, wheat and high-moisture corn that have fast rates of ruminal starch digestion generally cause the most problems. For example barley, wheat flour, oats and steam flaked corn all have ruminal starch availability greater than 85%. Guidelines for diets for dairy cattle producing more than 35-40kg of milk suggest neutral detergent fibre of 25-30% of the diet, with 75% of that from forage, non-structural carbohydrate levels of 35-40% and starch of 30-40% (Nocek 1997).

Acute acidosis is characterised by a precipitous decrease in ruminal pH with a high concentration of ruminal lactic acid (50-100mM). The ruminal microbial population undergoes a significant shift, with an increase in gram-positive lactic-acid producing bacteria, specifically *Streptococcus bovis* and *Lactobacillus* species. The falling pH leads to the death of gram-negative bacteria and the reduction or complete disappearance of ciliated protozoa. The shift in the fermentation pattern to lactate production is associated with decreased volatile fatty

acid (VFA) production. Systemic changes include decreased blood pH and bicarbonate and increased blood D and L-lactate. Acute acidosis can cause significant impairment of physiological functions such as ruminal stasis and dehydration, eventually leading to coma and death. Even if the animal survives, it may never completely recover.

5   Chronic acidosis has much more subtle clinical signs. The animals remain alert and consume feed, but may look 'off-colour'. The fall in rumen pH to below 5.5 is due to a general increase in fermentation within the rumen leading to greater production of VFAs. The increase of VFAs in the rumen is very highly correlated with increases in the blood, but blood pH does not change significantly. Total ciliated ruminal protozoa decline due to the falling pH, with

10   species differences in rate, but do not disappear entirely. Total viable bacterial counts increase over time, including increased amylolytic bacteria. However the overwhelming rise in S. bovis and Lactobacillus species seen in acute acidosis does not occur. While the rate of lactate production rises transiently after feeding the lactate is utilised immediately in production of VFAs, and does not accumulate in the rumen. Specifically the symptoms of

15   chronic acidosis are a fall in ruminal pH to 5.0 - 5.5 without significant lactic acid accumulation.

<u>Summary of symptoms of acute and chronic acidosis</u>

	Normal	Chronic acidosis	Acute acidosis
Rumen pH	>6.0	5.5 - 5.0	<5.0
VFAs	~100mM	up to 200mM	reduced
lactate concentration	up to 5mM	up to 5mM	>50mM
glucose	negligible	negligible	>10mM
Protozoa		much reduced	dead
Bacteria		increased	increased S. bovis and Lactobacillus sp.

Rumen acidosis is associated with many secondary conditions that can have a

20   significant impact on livestock animal performance, i.e. reduction in the feed conversion to meat and/or milk . Milk quality can also suffer in association with acidosis. Irreversible damage to the ruminal epithelium occurs at a rumen pH below 5.5, causing hyperkeratosis, papillary clumping and rumenitis of the ruminal epithelium. The animals have reduced appetite and performance due to impaired nutrient absorption, resulting in reduced weight

25   gain in beef cattle and decreased milk yield and quality in dairy cattle. Other effects are laminitis, intermittent diarrhoea, poor appetite and cyclic feed intake, a high herd cull rate for poorly defined health problems, poor body condition and abscesses without obvious causes. Chronic laminitis is one of the most consistent clinical signs, with ridges in the dorsal hoof wall, sole ulceration, white line lesions, sole haemorrhages and misshapen hooves. On

average, farmers report that 25% of animals in UK dairy herds are lame, and the true incidence of chronic laminitis is likely to be higher as it does not always produce detectable lameness. Liver abscesses are known to be linked with acidosis, and in most feedlots the incidence of liver abscesses averages from 12% to 32% of slaughtered cattle, and is a major

5   cause of liver condemnation. Liver abscesses are not necessarily diagnosed while the animal is alive, but have a deleterious effect on their performance and general health. Animals may also have depressed immune function, a high incidence of respiratory diseases and reduced fertility rates. Most dairy herds with a chronic acidosis problem have an annual herd turnover rate of greater than 45%, or an annual cull rate greater than 31%. The reasons for culling are

10   usually poorly defined. (Nocek 1997, Nordlund 1995, Nagaraja 1998, Stock and Britten 1998, AnimalPharm 1999, Kay 1969, McManus 1977).

Another problem which can be seen with high-yielding dairy cows fed with a high carbohydrate and/or low roughage diet is the acidosis-related "low milk fat syndrome". As the pH in the rumen falls, the pattern of fermentation shifts towards producing more propionate

15   and less acetate and butyrate. As approximately half of milk fat is produced from acetate and butyrate, this results in a drop in the milk fat content. (AT Chamberlain & JM Wilkinson, Feeding the Dairy Cow, Chalcombe Publications, UK, 1996).

Rumen acidosis and related problems are estimated to cost the livestock industry more than $1 billion per annum due to lost performance.

20   Recommended treatments for acute acidosis include administration of a mixture of sodium bicarbonate, formaldehyde, magnesium oxide and charcoal to kill rapidly dividing bacteria. (NebGuide G91-1047-A). Buffers are widely used (Horn 1979, Kennelly 1999), but do not seem efficacious enough to satisfy the livestock industry. Palatability of most buffers is low, and requires careful management to avoid reduced feed intake. Ionophore antibiotics

25   such as monensin, lasalocid and salinomycin are generally effective against gram-positive bacteria, including the major ruminal lactate-producing bacteria, S. bovis and Lactobacillus species (Burrin and Britton 1986, Coe 1999, Nagaraja 1985). They are therefore effective at preventing acute acidosis on transfer to high concentrate diets when cattle first reach the feedlot or following calving. They also act to reduce total VFA production in cattle with chronic

30   acidosis, and therefore stabilise rumen pH. However ionophores also decrease food intake. Other antibiotic classes have also been shown to prevent or ameliorate acute acidosis, including virginiamycin in sheep (Thorniley et al 1998), and the sulphur-containing peptide antibiotic thiopeptin, which is particularly effective against S. bovis (Armstrong 1984). However, sustained use of antibiotic feed additives is no longer seen as an appropriate

35   management tool (for review see: The use of drugs in food animals: benefits and risks, 1999). Probiotic control has been demonstrated with a number of species, including Selenomonas ruminantium subsp. lactolytica strain JDB201 (Wiryawan et al 1995), the lactate utilizer

Megasphaera elsedenii (Das, Kung and Hession 1995), and in more general terms patent WO 96/17525. The latter also claims enzymes that increase degradation of starch or fibre. Other proposed, but not commercialised, treatments include use of bacteriocins (Teather and Forster 1998), and the economically unviable manipulation of ruminal fermentation with organic acids (Martin, 1988, Martin et al. 1999).

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10. University of Nebraska, Lincoln NebGuide G91-1047-A http:/www.inar.unl.edu/pubs/ AnimalDisease/g1047.htm

There is a general need for a safe effective treatment for rumen acidosis;

especially chronic and/or acute rumen acidosis;

especially in ruminants such as cattle and sheep;

especially in lactating ruminants such as cattle and sheep;

which can preferably be administered easily, such as with food or drink;

which preferably is non-antimicrobial;

preferably which is palatable to the animal;

preferably which is active only in the rumen and has no systemic effects;

which preferably does not present any residues in meat and/or milk, and

which preferably does not require a withholding period;

which is preferably non-toxic to animal and feed handlers (manufacturer and farmer);

and/or which preferably can stabilise the rumen fermentation, thus preventing

5   excessive reductions in pH and maintaining VFA proportions such that milk fat production is not adversely affected.

We have discovered that certain inhibitors of bacterial α-amylase and/or α-glucosidase can be used to reduce ruminal pH in an effective way which should be useful in the treatment of both chronic and acute acidosis and related conditions.

10   By "inhibitor" herein is meant individual agents and mixtures of agents which have inhibitory activity, including fermentation broth products mentioned below.

One aspect of the invention is the use of an effective inhibitor of bacterial α-amylase and/or α-glucosidase in the manufacture of a composition for the treatment of acidosis. Of particular interest are inhibitors of amylases/glucosidases present in ruminal bacteria, such as

15   those mentioned hereinafter.

A further aspect of the invention is a method of treatment of acidosis which comprises administration of an effective amount of an inhibitor of bacterial α-amylase and/or α-glucosidase to an animal.
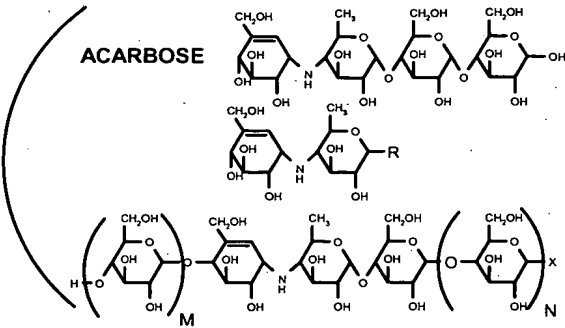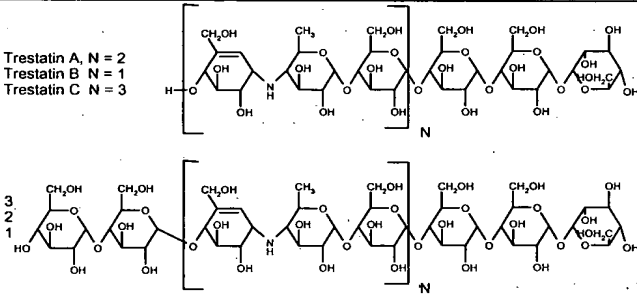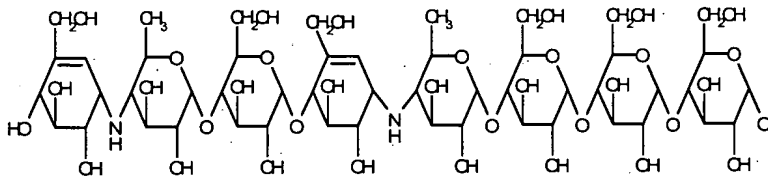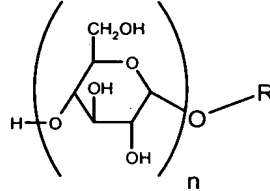
A further aspect of the invention is a formulation suitable for the treatment of acidosis

20   in an animal which comprises an inhibitor of bacterial α-amylase and/or α-glucosidase.
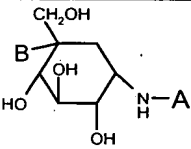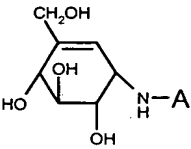
Further aspects of the invention are as defined in the claims.

Preferably the inhibitor of bacterial α-amylase and/or α-glucosidase has an IC_{50} of $10^{-3}M$ or less, more preferably $10^{-4}M$ or less, yet more preferably $10^{-5}M$ or less, in the rumen amylase and glucosidase screens described herein.
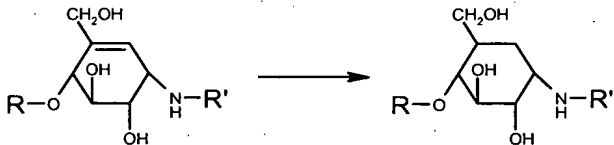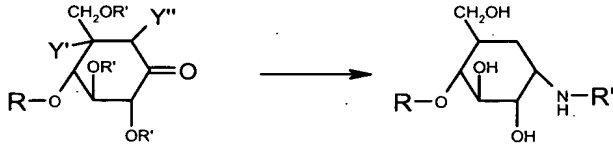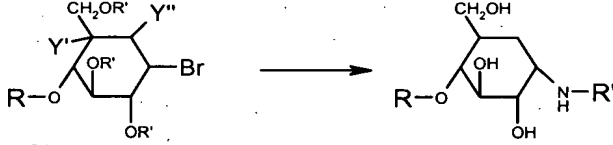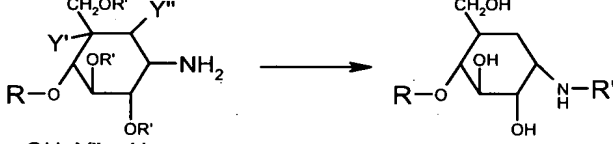
25   Preferably the amylase and/or α-glucosidase inhibitor has low antimicrobial activity, more preferably with a MIC value of more than 50μg/ml in the tests described herein, yet more preferably more than 100μg/ml.

A preferred group of α-amylase and/or α-glucosidase inhibitors include the substances disclosed below and simple analogues thereof, including in the Examples below,

30   which are found to be effective in the screens mentioned below (NB All references mentioned herein are hereby incorporated in their entirety) :

Amylostatins and semisynthetic analogues defined as "acarbose and higher homologues"*	Compounds disclosed generically and specifically in GB1,482,543; US 4,175,123; and in *Agric. Biol. Chem.*,46 (7), 1941-1945, 1982
Trestatin A, N = 2 / Trestatin B N = 1 / Trestatin C N = 3 Ro 09-0766, N = 3 / Ro 09-0767, N = 2 / Ro 09-0768, N = 1	Trestatins, e.g. those described in : *J. Antibiotics* 36: 1157-1165, (1983) *J. Antibiotics* 36: 1166-1175, (1983) And compounds described in *J. Antibiotics* 37(2): 182-186, (1984)
V-1532	V-1532 described in J.Mol. Biol. *260, 409-421, (1996)*
n = 0-4 R = 4 definitions of N containing moeities	compounds described in *Chem. Pharm. Bull* 47(2), 187-193 (1999); JP2000044589A
Amylase inhibitor SA-I	SA-I Described in *Agric. Biol. Chem,* 41(11) 2221-2228 (1977)

Extract from *Streptomyces* Strain DMC-72	Described in *Kor. J. Mycol.* Vol 13, No.4, 203-212, (1985)
	Compounds disclosed in JP159657; ES8800955A; WO8605094A; and EP194794A
Trestatin sulphate salts	EP-301-400-A
Pseudo-oligosaccharide from *Streptomyces sp.*FH 1717 (DSM 3006)	see EP-173950A
	compounds disclosed in EP-49981
	amylase inhibitors disclosed in Angew. Chem. Int. Ed. 20 , 744-761, (1981).
Example 7, Fraction 21 compound	see below
Example 8 compound	see below
Iso-acarbose, and B-acarbose and related structures described in --	*Tetrahedron Letters,* Vol. 37, No14, p2479-2482, (1996) *Archives of Biochemistry and Biophysics*, Vol 371, No.2, p277-283, (1999) *J. Chem. Soc. Chem. Commun.* No.9, p605-606 (1988)

Derivatives of the above compounds transformed as follows: 1. All above compounds with a valieneamine moiety can be transformed into the saturated analogue, produced by a reduction process described for example in EP-67356	

2. The chain extended, viz.:
 	
where Y' = OH, Y" = H;
or Y' = Y" = H;
or Y' + Y" = a bond
	see
	
	EP-240175-A

where Y' = OH, Y" = H;
or Y' = Y" = H;
or Y' + Y" = a bond
	see
	
	CH-648-326-A

where Y' = OH, Y" = H;
or Y' = Y" = H;
or Y' + Y" = a bond
	see
	
	EP-89812-A

Other derivatives incorporating the the valineamine core moiety, and of all compounds mentioned above, specifically acarbose, higher homologues thereof, trestatins, and V-1532, and the valeineamine compounds given by their registry numbers below* can be made by methods described herein.	
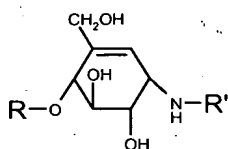
certain compounds with the moiety shown above appear in Chemical Abstracts* with the Registry Numbers (RN) shown below.

	1	RN	257941-10-9 REGISTRY
5	2	RN	257936-25-7 REGISTRY
	3	RN	250161-57-0 REGISTRY
	4	RN	244195-46-8 REGISTRY
	5	RN	227087-68-5 REGISTRY
	6	RN	223611-34-5 REGISTRY
10	7	RN	223608-57-9 REGISTRY
	8	RN	223608-52-4 REGISTRY
	9	RN	221371-17-1 REGISTRY
	10	RN	211247-58-4 REGISTRY
	11	RN	211247-57-3 REGISTRY
15	12	RN	211247-56-2 REGISTRY
	13	RN	211247-54-0 REGISTRY
	14	RN	211239-26-8 REGISTRY
	15	RN	211237-50-2 REGISTRY
	16	RN	207681-89-8 REGISTRY
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	18	RN	194539-38-3 REGISTRY
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	24	RN	194539-11-2 REGISTRY
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RN. 121624-16-6

RN 117193-65-4

$$RO-\text{cyclohexane ring with substituents: } OH, OH, NHR_1, OH, HO$$
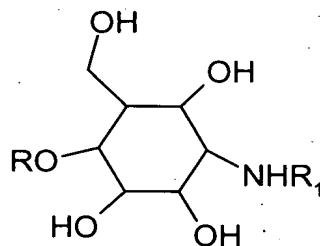
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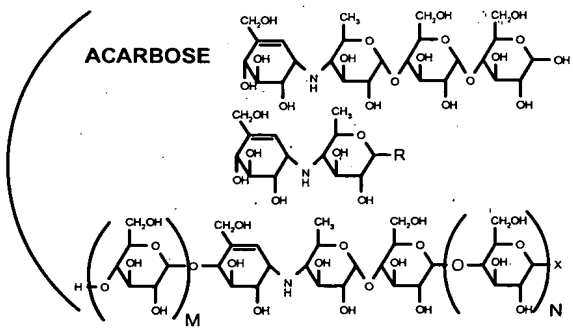RN 81739-22-2

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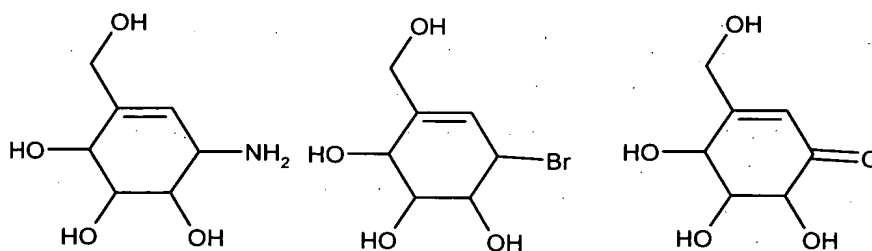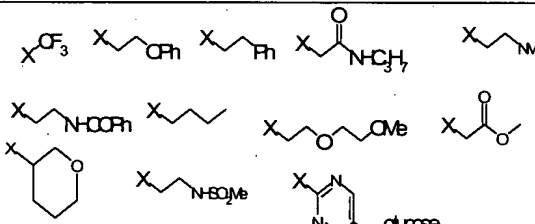
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RN 39318-73-5

RN 33034-94-5

20   By "acarbose and the higher homologues thereof" is meant the amylostatins of the formula given below, and mentioned generically and specifically in British Patent no. GB 1,482,543; US Patent 4,175,123; and in *Agric. Biol. Chem.*,46 (7), 1941-1945, 1982, all of which are herein incorprated by reference in their entirety.

Amylostatins and semisynthetic analogues ACARBOSE defined as "acarbose and higher homologues"* compounds where M=0 and N=1, 2 or 3 are disclosed in GB 1,482,543; compounds where M=0 to 8, and the sum of M+N is 0 to 7; X in both cases is OR, SH, SR, NH_2, NHR, or NRR^1, where R is alkyl, alkenyl, cycloalkyl, aralkyl, aryl or heterocyclyl and is defined in the quoted patents.	Compounds disclosed generically and specifically in GB1,482,543; US 4,175,123; and in *Agric. Biol. Chem.*,46 (7), 1941-1945, 1982

In addition to the amylase and/or α-glucosidase inhibitor compounds mentioned above, certain derivatives of said compounds can be made following the types of chemical transformation disclosed in the tables and references below, depending on the suitability of the substrate, and which transformations are expected to result in further amylase- and/or

5 glucosidase-inhibiting substances.

Preferably the substrate for such transformation is selected from the amylostatin compounds (i.e "acarbose and higher homologues" mentioned above), and trestatin compounds, V1532, the fraction 21 compound from Example 7, the Example 8 compound, and the compounds shown below (or suitably protected derivatives thereof) :

10

PROCESS	LITERATURE REF. (e.g.)	EXAMPLES OF REACTING GROUPS.
Synthetic or biotransformation attachment of a saccharide unit or oligosaccaharide via a N, S or O atom	CH-648-326-A J.Chem. Soc. Perkin Trans. 1 (1982) 1, pp15 - 18 Carbohydrate Research (1978) 67, 2, pp 305 - 328 Carbohydrate Research (1997) 305, 3-4, pp 561 - 568 - and see also later biotransformation section	Any monosaccharide or oligosaccahride such as glucose, ribose, xylose, mannose, galactose, sucrose, etc. Any monosaccharide of 2-6 sugar monomer units linked via any O or S for thio-sugars or N for aza-sugars Any cyclitol such as those described in *Cyclitols and their derivatives*, Hudlicky T, (1993), VCH publishers, Inc., New York Also Glucose-O-benzene-OH (attached via any oxygen) and Glucose-O-benzene-O-glucose, i.e which can be produced by methods exemplified in *Agric. Biol. Chem.* 53,1433, (1989) Phytochemistry, *40, 1149, (1995)* US patent US-42346684
Alkylation of any N or O with epoxide	EP-49981	Epoxides described in EP-49981.
Alkylation of any N or O with alkyl-leaving group, i.e iodide, bromide, mesylate, tosylate etc.	EP-49981	

PROCESS	LITERATURE REF. (e.g.)	EXAMPLES OF REACTING GROUPS.
Subtitution of C-leaving group with alcohol or amine	CH-648-326-A	Where X = NH₂ or OH. + Any nitrogen containing saccharide derivative
Reductive alkylation of N	EP-49981	
Reductive amination of carbonyl	EP-240175-A	+ Any nitrogen containing saccharide derivative
Addition to carbonyl with organometallic species	Tetrahedron, Vol 51, No.33, 9063-9078, (1995), Bull. Soc. Chim. Fr.,134, 777-784, (1997).	(suitably protected)
Oxidation of alcohol	Synlett, (5), 617-619, (1999) Org. Lett, 1 (9), 1475-1478 (1999)	
Acylation. Note X = suitable leaving group, ie chloride, organic acid etc.	US-4,175,123	Where X = appropriate leaving group i.e -Cl, or RCO₂- etc

PROCESS	LITERATURE REF. (e.g.)	EXAMPLES OF REACTING GROUPS.
C-C double bond formation from carbonyl or lactol	*Tetrahedron Assymetry* 3(3), 451-8 (1992). *J.Org. Chem.* 61(11), 3594-3598, (1996)	

All the substances mentioned herein can be labelled e.g. with isotopes of certain atoms, as is well known in the art. Such isotopically-labelled substances are available by well-known methods in the art.

Preferred inhibitors include acarbose and higher homologues thereof, Trestatin A, Trestatin C, the compound of Fraction 21 of Example 7 below, Example 8 below, as well as the fermentation broth products mentioned below.

A preferred group of inhibitors are substantially pure single compound, or partially-purified fermentation or biotransformation product, inhibitors including acarbose and higher homologues thereof, Trestatin A, Trestatin C, the compound of Fraction 21 of Example 7 below, Example 8 below.

Especially preferred are acarbose and Trestatin C.

Some of the inhibitors may be made by biotransformation / fermentation, such as the methods described herein below.

Biotransformation / fermentation products

The cultures *Streptomyces conglobatus* ATCC31005, *Streptomyces coelicolor* subsp. *flavus* ATCC19894, *Streptomyces kursannovii* ATCC11912 and *Streptomyces lienomycini* ATCC43687 were obtained from the American Type Culture Collection (ATCC located at 10801 University Boulevard, Manassas, Virginia 20110-2209, U.S.A.). The cultures *Streptomyces* sp. KC672 isolated from a marine sediment in Suruga Bay, Japan and *Streptomyces* sp.CL45763 have been deposited in accordance with the Budapest Treaty at the National Collections of Industrial and Marine Bacteria Ltd. and assigned the accession numbers NCIMB41058 and NCIMB41057 respectively. (NCIMB is located at 23 St. Machar Drive, Aberdeen, U.K. AB24 3RY.). The depositor was Pfizer Central Research, Pfizer Limited, Ramsgate Road, Sandwich, Kent, CT13 9NJ, United Kingdom. Pfizer Ltd. is a wholly-owned subsidiary of Pfizer Inc. 235 East 42nd Street, New York, New York, USA.

In addition, mutant strains of *Streptomyces conglobatus* ATCC31005, *Streptomyces coelicolor* subsp. *flavus* ATCC19894, *Streptomyces kursannovii* ATCC11912, *Streptomyces lienomycini* ATCC43687, *Streptomyces* sp. KC672 and *Streptomyces* sp.CL45763 can be

used: Such mutant strains can be obtained spontaneously, or by the application of known techniques, such as exposure to ionising radiation, ultraviolet light, and/or chemical mutagens such as N-methyl-N-nitrosourethane, N-methyl-N'-nitro-N-nitrosoguanadine, ethyl methyl sulphate etc. Genetically transformed and recombinant forms include mutants and genetic variants produced by genetic engineering techniques, including for example recombination, transformation, transduction, protoplast fusion etc.

Fermentation of the cultures of *Streptomyces conglobatus* ATCC31005, *Streptomyces coelicolor* subsp. *flavus* ATCC19894, *Streptomyces kursannovii* ATCC11912, *Streptomyces lienomycini* ATCC43687, *Streptomyces* sp. KC672 and *Streptomyces* sp.CL45763 can be carried out using standard procedures well known in the art for filamentous bacteria of the genus *Streptomyces*. For example growth of the organism may take place on suitable solid medium or aqueous liquid medium under aerobic conditions in the range 24 to 35°C using suitable sources of carbon, nitrogen and trace elements such as iron, zinc, manganese for 2 to 30 days.

Use is made of the following fermentation media.

<u>AP5-H Production Medium</u>

Corn starch (Hidex)	80g
Yeast extract (Oxoid)	5g
K_2HPO_4	1g
$MgSO_4.7H_2O$	1g
Glutamic acid	1g
$FeSO_4.7H_2O$	0.01g
$ZnSO_4.2H_2O$	0.001g
$MnSO_4.2H_2O$	0.001g
$CaCO_3$	7g
Tap water	1l
NaOH	To pH 7.0

<u>½ strength MECO Medium</u>

Glucose	5g
Acid Hydrolysed Starch (Hidex[TM], Japan)	10g
Casitone[TM] (Difco - nitrogen source)	2.5g
Yeast extract (Oxoid[TM])	2.5g
Wheat embryo (Sigma)	2.5g
Calcium carbonate	2.0g
Demineralised water	1l
NaOH	To pH 7.0

<u>Modified ANG-3 medium</u>

Soluble starch	20g
Glucose	100g
Soya Flour (Trusoy™)	10g
NaNO$_3$	2g
K$_2$HPO$_4$	1g
MgSO$_4$.7H$_2$O	0.5g
KCl	0.5g
FeSO$_4$.7H$_2$O	0.01g
MOPS buffer (Sigma)	20g
Demin water	1l
NaOH	to pH7

All the media described can be supplemented with other starches, partially hydrolysed starches or soluble starches and or sugars such as D-xylose, D-ribose, D-maltose, D-maltotriose, D-sedoheptulose, D-trehalose, D-glucose and or nitrogen sources such as asparagine, aspartate and glutamine.

Example 1 – Preparation of a fermentation broth demonstrating rumen fluid α-amylase inhibitory activity from *Streptomyces conglobatus* ATCC31005

Streptomyces conglobatus ATCC31005 maintained on a ¼ strength ATCC172 agar slope was inoculated as a loopful of spores into two 300 ml Erlenmeyer flasks each containing 50mls of ½ strength MECO medium. They were then allowed to incubate for 7 days at 28°C, 200rpm on an Infors Multitron Shaker with 1" (2.5 cm) throw. At this point the broth was centrifuged at 3500 rpm and the supernatant removed from the mycelium. The α-amylase inhibitory activity was determined for the supernatant which is summarised in the table below.

Supernatant dilution into assay	1:1000	1:10000	1:100000
% inhibition flask 1	91	89	65
% inhibition flask 2	86	90	58

Example 2 – Preparation of a fermentation broth demonstrating rumen fluid α-amylase inhibitory activity from *Streptomyces* sp. CL 45763

Streptomyces sp. CL45763 maintained on an agar slope of Bacto ISP-3 was inoculated as a loopful of spores into four 300 ml Erlenmeyer flasks each containing 50mls of AP5-H medium. They were then allowed to incubate for 9 days at 28°C, 200rpm on an Infors Multitron Shaker with 1" (2.5 cm) throw. At this point the broths were combined, centrifuged at 3500 rpm, and then the supernatant removed from the mycelium. The α-amylase inhibitory activity for the supernatant was then determined which is summarised in the table below.

Supernatant dilution into assay	1:1000	1:10000	1:100000
% inhibition	79	77	53

Example 3– Preparation of a fermentation broth demonstrating rumen fluid α-amylase inhibitory activity from *Streptomyces coelicolor* subsp. *flavus* ATCC19894

5   *Streptomyces coelicolor* subsp. *flavus* ATCC19894 maintained on a ¼ strength ATCC172 agar slope was inoculated as a loopful of spores into ten 300 ml Erlenmeyer flasks each containing 50mls of AP5-H medium. They were then allowed to incubate for 4 days at 28°C, 200rpm on an Infors Multitron Shaker with 1" (2.5 cm) throw. At this point the broths were centrifuged at 3500 rpm and the supernatant removed from the mycelium. The α-amylase inhibitory activity was determined for the combined supernatants which is

10   summarised in the table below.

Supernatant dilution into assay	1:1000	1:10000
% inhibition flask 1	66	35

Example 4– Preparation of a fermentation broth demonstrating rumen fluid α-amylase inhibitory activity from *Streptomyces kursannovii* ATCC11912

Streptomyces kursannovii ATCC11912 maintained on a ¼ strength ATCC172 agar

15   slope was inoculated as a loopful of spores into two 300 ml Erlenmeyer flasks each containing 50mls of AP5-H medium. They were then allowed to incubate for 5 days at 28°C, 200rpm on an Infors Multitron Shaker with 1" (2.5 cm) throw. At this point the broths were centrifuged at 3500 rpm and the supernatant removed from the mycelium. The α-amylase inhibitory activity was determined for each supernatant which is summarised in the table

20   below.

Supernatant dilution into assay	1:1000	1:10000	1:100000
%inhibition Flask 1	85	73	27
%inhibition Flask 2	85	70	30

Example 5– Preparation of a fermentation broth demonstrating rumen fluid α-amylase inhibitory activity from *Streptomyces lienomycini* ATCC43687

Streptomyces lienomycini ATCC43687 maintained on a ¼ strength ATCC172 agar

25   slope was inoculated as a loopful of spores into three 300 ml Erlenmeyer flasks each containing 50mls of modified ANG-3 medium. They were then allowed to incubate for 5 days

at 28°C, 200rpm on an Infors Multitron Shaker with 1" (2.5 cm) throw. At this point the broths were centrifuged at 3500 rpm and the supernatant removed from the mycelium. The α-amylase inhibitory activity was determined for each individual supernatant which is summarised in the table below.

Supernatant dilution into assay	1:1000	1:10000	1:100000
% inhibition flask1	87	70	41
% inhibition flask2	85	75	41
% inhibition flask3	85	76	47

Example 6– Preparation of a fermentation broth demonstrating rumen fluid α-amylase inhibitory activity from *Streptomyces* sp. KC672

Streptomyces sp. KC672 maintained on a ¼ strength ATCC172 agar slope was inoculated as a loopful of spores into two 300 ml Erlenmeyer flasks each containing 50mls of AP5-H medium. They were then allowed to incubate for 7 days at 28°C, 200rpm on an Infors Multitron Shaker with 1" (2.5 cm) throw. At this point the broths were centrifuged at 3500 rpm and the supernatant removed from the mycelium. The α-amylase inhibitory activity was determined for the supernatants which is summarised in the table below.

Supernatant dilution into assay	1:1000	1:10000
%inhibition flask 1	54	21
%inhibition flask 1	49	22

Example 7 – Isolation of a rumen fluid α-amylase inhibitor from *Streptomyces conglobatus* ATCC31005

A loopful of spores of *Streptomyces conglobatus* ATCC31005 maintained on ¼ strength ATCC172 agar was inoculated into two 300ml Erlenmeyer flasks, each containing 50 ml of AP5-H medium. After 24 hours incubation at 28°C, 200rpm on an Infors Multitron Shaker with 1" (2.5 cm) throw, these flasks were used to inoculate two 5 litre minijars (Electrolab™, Gloucester, U.K) each containing 3.5 litres each of AP5-H medium. These broths were incubated at 28°C with an aeration of 3l/min and stirring at 300rpm for 6 days. At harvest the broths were centrifuged at 2500 rpm and the supernatants decanted. They were each stirred twice for 45 minutes with 86g of charcoal and then filtered through a 500g bed of Arbocel™. The pH at this stage was pH7. Each carbon cake was extracted twice with 860mls of aqueous acetone (1:1) maintaining the pH between 2 and 3 by the addition of concentrated hydrochloric acid. The four aqueous acetone extracts were partially evaporated, combined

and lyophilised to give 40g of a brown solid. This solid was then dissolved in 500mls of deionised water and applied to a column of 750mls Amberlite IR120 (H^+ form) at a flow rate of 2ml/min. The column was then washed with 1l. of water and then eluted with 5N ammonia solution collecting 50ml fractions. Fractions 13 to 28 were lyophilised to give 1.4g of a dark brown powder. This was then dissolved in 15mls of water, filtered and the resulting filtrate diluted with 5mls of acetonitrile. This was injected in 2ml volumes on to a Cosmosil NH_2-MS column (20x250mm) and eluted at 20mls/min with acetonitrile water (60:40). Fractions were collected every 30 seconds and analysed by LC-MS using a Finnegan AQA™ instrument. Fractions containing $M+H^+$970 were combined and dried down to give a gum solid, 70mgs. The solid was then dissolved in two mls of water, filtered and injected in two halves on to an Waters Aqua™ 5 micron 125A column (21x150mm), using a Waters Delta Prep™ 4000 system with diode array detection. Fractions were collected and two, both fraction 21, were combined containing the $M+H^+$970 peak resulting in a white solid, 6.5 mg.

Accurate mass data was collected on a Bruker Apex II FT-ICR-MS 4.7 T instrument where the sample, dissolved in methanol/water/acetic acid (50:50:1) at approx 0.5 mgs/ml, was introduced into an Analytica electrospray source by direct infusion at 4µl/min.

m/z (ESI, FTMS) $[M+H]^+$ = 970.3601, $C_{37}H_{64}NO_{28}$ requires 970.3609

m/z (ESI, FTMS) $[M+Na]^+$ = 992.3452, $C_{37}H_{63}NO_{28}Na$ requires 992.3429

The NMR (proton, carbon-13, TOCSY, HSQC and HMBC) and mass spectra of this fraction 21 compound, also known as "6942/99/1" are consistent with the structure shown below.
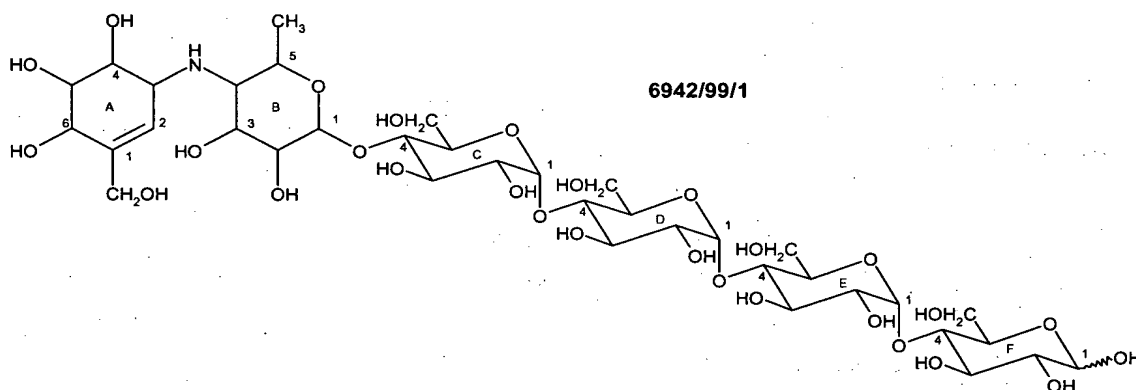
6942/99/1

The compound shown above has been disclosed in GB patent 1 482 543 and (Ag.Biol. Chem. 46(7) (1982) 1941).

Table : ^1H and ^{13}C NMR chemical shifts of 6942/99/1 (δ in ppm relative to internal dioxane)

Position	δ_H	multiplicity	J (Hz)	δ_C
A1-CH$_2$OH	4.09/4.20	2 x d	14.2	64.5

Position	δ_H	multiplicity	J (Hz)	δ_C
A1	-	-	-	141.9
A2	5.87	d, br	5.3	126.6
A3	3.51	t, br	~5	58.9
A4	3.63	m	-	73.9
A5	3.73	m	-	75.9
A6	4.01	d, br	6.9	74.2
B1	5.28	m	3.6	102.8
B2	3.57	m	-	75.6
B3	3.58	m	-	
B4	2.45	t	9.7	67.8
B5	3.72	m	-	72.6
B6-Me	1.31	d	6.4	20.2
Rings C-E: 1	~5.37	d	4.0	~102.4
2	~3.60	dd	9.5, 4.0	~74.4
3	~3.93	t	~9.5	~76.2
4	~3.62	t	~9.5	~79.9
5	~3.81	m	-	~74.2
6-CH$_2$	~3.79/3.83	m	-	~63.4
α-F1	5.20	d	3.9	94.9
α-F2	3.54	dd	~9.5, 3.9	
Position	δ_H	multiplicity	J (Hz)	δ_C
α-F3	3.94	t	~9.5	76.2
α-F4	3.62	t	~9.5	~79.9
α-F5	3.91	m	-	72.8
α-F6-CH$_2$	~3.8	m	-	~63.4
β-F1	4.63	d	7.9	98.8
β-F2	3.25	dd	7.9, 9.5	76.9
β-F3	3.74	t	9.5	79.1
β-F4	3.63	dd	9.5, 8.3	~79.9
β-F5	3.56	m	-	77.5
β-F6-CH$_2$	~3.87	m	-	~63.4

The "Fraction 21 compound" was found to have an inhibiting effect in the amylase screen mentioned herein.

Example 8 – Isolation of a rumen fluid α-amylase inhibitor from *Streptomyces conglobatus* ATCC-31005

The AP5-H Production Medium mentioned above was used as fermentation medium.

Streptomyces conglobatus ATCC31005 maintained on a ¼ strength ATCC172 agar
5   slope was inoculated as a loopful of spores into two 300ml Erlenmeyer flasks each containing 50mls of AP5-H medium. They were then allowed to incubate for 24 hours at 28°C, 200rpm on an Infors Multitron Shaker with 1" throw. At this point the inoculum was transferred into a 3 litre Fernbach flask containing 1 litre of AP5-H medium and incubated for a further 24 hours under the same conditions as described for the Erlenmeyer flasks. This inoculum was then
10   transferred to 20litres of AP-5H medium which had previously been sterilised in a 30 litre New Brunswick Micros™ stainless steel fermenter. The broth was then agitated at 300rpm at 28°C with 20l/min air for 112 hours and then harvested.

The harvested broth was centrifuged using a Carr Powerfuge™ at 20 000G. To the supernatant, at a natural pH of 7.8, was added 500g of activated decolourising charcoal
15   (Aldrich 16155-1) and the mixture stirred for 16 hours. Following filtration through a filter aid, such as Arbocel™, the supernatant was treated with a further 500g charcoal, for 1 hour in the same manner. The combined charcoal cakes were washed with aqueous methanol (10L 1:1) and then extracted twice with aqueous acetone (10L 1:1) by stirring for 1 hour followed by filtration through filter aid. Following partial rotary evaporation and freeze drying, 98.7g of
20   biologically active material were obtained.

This material was dissolved in 1800ml demineralised water and loaded onto a column of 3.5L Amberlite IR 120(H) ™ at a rate of 5ml/minute. Following a water wash (2L) the product was eluted with 1L aliquots of 5N ammonia solution. Following freeze drying, the most potent fractions (5 to 8) were combined to give 10.8g of brown solid.

25   1.1g of this material was purified by chromatography, in five equal injections, using a Waters Delta Prep 4000™ chromatography system, a 250 x 21.2mm CromasilNH$_2$ (ex Phenomenex) and a gradient from 67% acetonitrile 33% water to 50/50 at 20 minutes at a flow rate of 24 ml/minute. 12ml fractions were collected.

The fractions containing the peak of interest (31 to 35 from each run) were combined
30   to give 138mg white solid. This material was chromatographed again, this time using a 150 x 21.2mm Aqua column (ex Phenomenex) and a gradient from 100% water to 90% water 10% acetonitrile over 15 minutes at a flow rate of 21.2 ml/minute. Fractions were collected at half minute intervals. A total of 43mg of desired product, I was obtained from fractions 22 and 23.

The observed data are consistent with the following structure, referred to herein as
35   the "Example 8 compound":
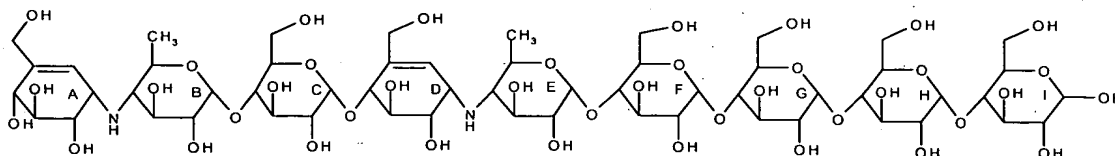
M/z (ESI, FT-MS) $[M+H]^+$ = 1435.546 corresponding to a molecular formula of $C_{56}H_{95}N_2O_{40}$ (+/- 3.028ppm).

M/z (ESI, FT-MS) $[M+Na]^+$ = 1457.528 corresponding to a molecular formula of $C_{56}H_{94}N_2O_{40}Na$ (+/- 1.914ppm).

All NMR data given below were recorded on a Varian Innova 600 MHz machine at $10^{\circ}C$ in D_2O using a 3mm probe.

Position	H	Multiplicity	J(Hz)	C
A1-CH2OH	4.09/4.21	2 x d	14.2	64.2
A1	-	-	-	141.7
A2	5.88	d,br	5.3	126.2
A3	3.52	t,br	~5	58.8
A4	3.64	m	-	~73.9
A5	3.74	m	-	~75.9
A6	4.01	d,br	7.2	~73.7
B1	5.33	m	3.6	~102.2
B2	3.57	m	-	75.5
B3	3.58	m	-	?
B4	2.45	t	9.7	67.7
B5	3.72	m	-	72.2
B5-Me	1.31	d	6.4	20.0
C1	5.36	d	3.8	100.1
C2	3.59	dd	-	73.8
C3	3.89	t	-	76.2
C4	3.61	t	-	~79.3
C5	3.88	m	-	~74.2
C6-CH2	3.82	m	-	~63.1
D1-CH2OH	4.09/4.21	2 x d	14.1	64.7
D1	-	-	-	139.1
D2	5.95	d.br	4.1	128.9
D3	3.52	t,br	~5	57.7
D4	3.82	m	-	71.9

Position	H	Multiplicity	J(Hz)	C
D5	4.15	dd	8.2,5.3	73.3
D6	4.21	d.br	6.9	78.4
E1	5.33	m	3.6	~102.2
E2	3.57	m	-	75.6
E3	3.58	m	-	?
E4	2.45	t	9.7	66.7
E5	3.72	m	-	72.2
E5-Me	1.31	d	6.4	20.0
Rings F-H:1	~5.40	d	3.8	~102.2
2	~3.59	dd	9.5/d.0	~75.2
3	~3.93	t	~9.5	~76.1
4	~3.65	m	~9.5	~79.1
5	~3.82	m	-	~73.8
6-CH2	~3.79/3.83	m	-	~63.1
α-I1	5.21	d	3.8	94.9
α-I2	3.54	dd	~9.5,3.9	
α-I3	3.94	t	~9.5	76.2
α-I4	3.62	t	~9.5	~79.9
α-I5	3.91	m	-	72.8
α-I6-CH2	~3.8	m	-	~63.4
β-I1	4.63	d	7.9	98.7
β-I2	3.25	dd	7.9,9.5	76.9
β-I3	3.74	t	9.5	79.1
β-I4	3.63	dd	9.5,8.3	~79.9
β-I5	3.56	m	-	77.5
β-I6-CH2	~3.87	m	-	~63.4

NB where a "?" appears in the table above, there was severe signal overlap meaning that an unambiguous assignment could not be made

Modifications to acarbose and other related α-amylase and/or α-glucosidase inhibitors

Biotransformations

Microbial biotransformation

Microbial whole organisms capable of glycosylation of acarbose or other related α-amylase and/or α-glucosidase inhibitors could be used to give increased α-amylase and/or α-glucosidase inhibitory activity, which include Bacillus subtilis ATCC55060[1],

Saccharopolyspora erythrae ATCC11635[2] and a blocked mutant of *S.avermitilis* ATCC53567[3]. Other organisms which can glucosidate include *Cunninghamella* sp. NRRL5695[14] and *Beauvaria bassiana* DSM 875 and DSM 1344[15]. Moreover the microbial directed biosynthesis of acarbose by an *Actinoplanes* sp. CBS 793.96[4] fed with rutin could also be used with other related α-amylase and/or α-glucosidase inhibitor producing organisms. These may give analogues of acarbose or related acarbose like homologues which could also demonstrate increased α-amylase and/or α-glucosidase inhibitory activity.

Moreover microorganisms capable of O-acylation[16], oxidation (incl. epoxidation[17] and ketone[18] formation), hydroxymethylation[19] O-methylation[20], etc. can also be used to make new analogues of acarbose and related analogues which could also demonstrate increased α-amylase and/or α-glucosidase inhibitory activity.

Crude, partially purified and purified enzyme biotransformations

Enzymatic methods of glycosylation can be used to synthesise or modify oligosaccharides. Specific protection and deprotection of hydroxyl groups is not required and the enzymes only transfer to one or two hydroxyl groups. This often leads to fewer reaction steps and simpler purification procedures.

Transglycosylation of *Bacillus stearothermophilus* maltogenic amylase (BSMA) with acarbose and various acceptors have been used, where the enzyme was an *Escherichia coli* transformant carrying the BSMA gene.[5] Here it was observed that the BSMA cleaved the first glycosidic bond of acarbose to give the pseudotrisaccharide (PTS) and then added on a glucose unit at the α (1→6) position to give isoacarbose, where acarbose itself has an α (1→4) linkage at the terminal glucose. The addition of a number of different carbohydrates to the digest gave transfer products in which the PTS was primarily attached α (1→6) to D-glucose, D-mannose, D-galactose and methyl α-D-glucopyranoside. With D-fructopyranose and D-xylopyranose, PTS was linked at α (1→5) and α (1→4) respectively. α-α Trehalose and maltitol *both* gave two major products with PTS linked α (1→6) and α (1→4) to the glucopyranose residue. PTS was primarily transferred to C-6 of the nonreducing residue of maltose, cellobiose, lactose and gentiobiose. Sucrose gave PTS linked α (1→4) to the glucose residue. Raffinose gave two major products with PTS linked α (1→6) and α (1→4) to the D-galactopyranose residue. Maltotriose gave two major products with PTS linked α (1→6) and α (1→4) to the nonreducing end glucopyranose residue. Xylitol gave PTS linked α (1→5) as the major product and D-glucitol gave PTS linked α (1→6) as the only product. All these examples may show improved α amylase inhibitory activity.

Other groups of enzymes can be used to produce glycosidated analogues of acarbose or other amylase and/or α-glucosidase inhibitors that have accessible sugar or hydroxyl groups. Enzymatic preparations that may be used include α- and β- galactosidase,

α- and β- mannosidase, β-N-acetylglucosaminidase, β-N-acetylgalactoaminidase, and α-L fucosidase.[6]Glycosidation can take place at either end of the valienamine or cyclitol unit of acarbose and experience shows that the glycosyl transfer is preferred to take place at the non reducing terminal monosaccharide unit of substrates.[7] Studies using endo glycosidases may lead to branched structures. The enzyme preparations described can be microbially derived e.g *Aspergillus niger, A.terreus, A.oryzae, Bacillus circulans, B.stearothermophilus, Coccobacillus,* or insect juice e.g snail, or plant derived e.g apples, mushrooms, alfalfa seeds, defatted almond meal etc.[6]

Glycosyltransferases can also be used to glycosylate acarbose and related analogues demonstrating α-amylase inhibitory activity but are much rarer enzymes.[8] Many of these glycosyltransferases have been cloned. They are often referred to as being rather stringent to the distal one to two saccharide moieties and are also very specific to the glycosyl donor. They can be persuaded to work with both unnatural donors and/or acceptors maintaining their advantages of strict regio and stereoselectivity and high yields. Only a few glycosyltransferases are readily available and most experiments have been carried out with galactosyltransferase Gal T.[9]

A special group of glycosyl transferases are cyclodextrin glucanotransferases (CGTase). These enzymes are produced by microorganisms and many are commercially available. They catalyse cyclodextrination of starch but also transfer one or more α- glucosyl units to various acceptors. They can be used for extending glycosides or for α- glucosylation of many compounds. CGTase from *B.stearothermophilus* was used for the transglucosylation of rutin where the glucosyl unit was extended by one or more glucose units.[10] A similar approach could be used for acarbose and related α- amylase and/or α-glucosidase inhibitors containing glucose units.

Another approach is to use glycogen phosphorylase which is the well known enzyme responsible for the formation or degradation of α (1→4) glucans. Phosphorylase requires an activated substrate such as the glucosyl phosphate ester. With this substrate a glucan chain, the primer unit, can be elongated by glucose units with the release of phosphate.[11]

Modification of acarbose and related α amylase and/or α-glucosidase inhibitors containing sugar units can also be made using selective hydrolyses with α amylase itself which can either cleave sugar units or transglycosylate[12,13]

For other modifications of the hydroxyl groups of sugar units acylases, esterases, lipases, hydrolases and dehydratases can also be used.

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Certain of the substances mentioned herein can exist in one or more geometric and/or stereoisomeric forms. The present disclosure includes all such individual isomers and salts and prodrugs thereof. Certain compounds mentioned herein could exist in more than one tautomeric form. Similarly certain compounds mentioned herein may have zwitterionic forms. It is to be understood that the disclosure embraces all such tautomers, zwitterions and their derivatives.

The disclosure includes veterinarily acceptable salts of the compounds mentioned herein, including the acid addition and the base salts thereof where appropriate. Suitable acid addition salts are formed from acids which form non-toxic salts and examples are the hydrochloride, hydrobromide, hydroiodide, sulphate, hydrogen sulphate, nitrate, phosphate, hydrogen phosphate, acetate, maleate, fumarate, lactate, tartrate, citrate, gluconate, succinate, benzoate, methanesulphonate, benzenesulphonate and p-toluenesulphonate salts. Suitable base salts are formed from bases which form non-toxic salts and examples are the aluminium, calcium, lithium, magnesium, potassium, sodium, zinc and diethanolamine salts. For a review on suitable salts see Berge et al, J. Pharm. Sci., 66, 1-19 (1977).

It will be appreciated by those skilled in the art that certain protected derivatives of compounds mentioned herein, which may be made prior to a final deprotection stage, may not possess the desired biological activity as such, but may, in certain instances, be transformed after administration into the body, for example by metabolism, to form compounds mentioned herein which are biologically active. Such derivatives are included in the term "prodrug". It will further be appreciated by those skilled in the art that certain moieties known to those skilled in the art as "pro-moieties", for example as described in "Design of Prodrugs" by H Bundgaard (Elsevier) 1985, may be placed on appropriate functionalities when such functionalities are present in compounds mentioned herein, also to form a "prodrug". Further, certain compounds mentioned herein may act as prodrugs of other

compounds mentioned herein. All protected derivatives, and prodrugs, of the compounds mentioned herein are included within the scope of the disclosure.

The skilled person will appreciate that certain substances mentioned herein can be made by methods other than those hereinbefore described, by adaptation of the methods herein described and/or adaptation of methods known in the art, for example the art described herein, or using standard textbooks such as

"Comprehensive Organic Transformations - A Guide to Functional Group Transformations", RC Larock, VCH (1989 or later editions),

"Advanced Organic Chemistry - Reactions, Mechanisms and Structure", J.March, Wiley-Interscience (3rd or later editions),

"Organic Synthesis - The Disconnection Approach", S Warren (Wiley), (1982 or later editions),

"Designing Organic Syntheses" S Warren (Wiley) (1983 or later editions), "Guidebook To Organic Synthesis" RK Mackie and DM Smith (Longman) (1982 or later editions);

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etc.,

and the references therein as a guide.

It is to be understood that the synthetic transformation methods mentioned herein are exemplary only and they may be carried out in various different sequences in order that the desired compounds can be efficiently assembled. The skilled chemist will exercise his judgement and skill as to the most efficient sequence of reactions for synthesis of a given target compound. For example, substituents may be added to and/or chemical transformations performed upon, different intermediates to those mentioned hereinafter in conjunction with a particular reaction. This will depend *inter alia* on factors such as the nature of other functional groups present in a particular substrate, the availability of key intermediates and the protecting group strategy (if any) to be adopted. Clearly, the type of chemistry involved will

influence the choice of reagent that is used in the said synthetic steps, the need, and type, of protecting groups that are employed, and the sequence for accomplishing the synthesis. The procedures may be adapted as appropriate to the reactants, reagents and other reaction parameters in a manner that will be evident to the skilled person by reference to standard

5   textbooks and to the examples provided hereinafter.

It will be apparent to those skilled in the art that sensitive functional groups may need to be protected and deprotected during synthesis of a compound of the invention. This may be achieved by conventional methods, for example as described in "Protective Groups in Organic Synthesis" by TW Greene and PGM Wuts, John Wiley & Sons Inc (1999), and

10   references therein. Functional groups which may desirable to protect include oxo, hydroxy, amino and carboxylic acid.   Suitable protecting groups for oxo include acetals, ketals (e.g. ethylene ketals) and dithianes. Suitable protecting groups for hydroxy include trialkylsilyl and diarylalkylsilyl groups (e.g. ' tert-butyldimethylsilyl, tert-butyldiphenylsilyl or trimethylsilyl) and tetrahydropyranyl.   Suitable protecting groups for amino include tert-butyloxycarbonyl, 9-

15   fluorenylmethoxycarbonyl or benzyloxycarbonyl.   Suitable protecting groups for carboxylic acid include C_{1-6} alkyl or benzyl esters.

The amylase and/or α-glucosidase inhibitors may be administered either alone or in combination with one or more agents used in the treatment (incuding prophylaxis) of disease or in the reduction or suppression of symptoms as appropriate for the treatment of acidosis

20   and related conditions. Examples of such agents (which are provided by way of illustration and should not be construed as limiting) include buffers, antibiotics including ionophores, probiotics, organic acids and bacteriocins, antiparasitics, eg fipronil, lufenuron, imidacloprid, avermectins (eg abamectin, ivermectin, doramectin), milbemycins, organophosphates, pyrethroids; antihistamines, eg chlorpheniramine, trimeprazine, diphenhydramine,

25   doxylamine; antifungals, eg fluconazole, ketoconazole, itraconazole, griseofulvin, amphotericin B; antibacterials, eg enroflaxacin, marbofloxacin, ampicillin, amoxycillin; anti-inflammatories eg prednisolone, betamethasone, dexamethasone, carprofen, ketoprofen; dietary supplements; eg gamma-linoleic acid; and emollients.

The amylase and/or α-glucosidase inhibitors can be administered alone but will

30   generally be administered in admixture with a suitable excipient, diluent or carrier selected with regard to the intended route of administration and standard pharmaceutical / veterinary / farming practice.

Advantageously for treatment of livestock animals such as sheep and cattle, the active agent can be administered orally using suitable standard methods such as mixed with

35   the animal's feedstuff, in the drinking fluid or via a bolus delivered directly to the rumen. For in-feed administration a concentrated feed additive or premix may be provided for mixing with

the normal animal feed. Additional physical and chemical stabilising agents may also be included to maintain or enhance the stability of the active agents in the said formulation.

The methods by which the active agent may be administered include oral administration by capsule, bolus, tablet or drench, or, alternatively, they can be administered by injection or as an implant into the rumen. Such formulations may be prepared in a conventional manner in accordance with standard veterinary practice.

For example, the active agent can be administered orally in the form of solutions, powders or suspensions, which may contain flavouring or colouring agents, for immediate-, delayed-, modified-, sustained-, pulsed- or controlled-release applications.

In addition to in-feed or in-drink administration with part of the cattle's normal diet, it is envisaged that the active agent could be separately administered between normal feeding and drinking, e.g. in the form of a palatable "treat" such as in a molasses-based formulation.

For aqueous suspensions and/or elixirs, the active agent may be combined with various sweetening or flavouring agents, colouring matter or dyes, with emulsifying and/or suspending agents and with diluents such as water, ethanol, propylene glycol and glycerin, and combinations thereof. Additional physical and chemical stabilising agents may also be included to maintain or enhance the stability of the active agents in the said formulation.

The active agent may also be delivered via a long-acting bolus formulation directly to the rumen, wherein the formulation device is retained within the ruminoreticular sac for prolonged periods of time to facilitate sustained release. Ruminal retention of the formulation device as described in this instance may be achieved using dense matrices or reservoirs based on aluminium or steel cylinders or pellets formed from a mixture of clay, drug and other ingredients.

The active agent may, in certain cases, also be administered parenterally, for example, intravenously, intra-arterially intraperitoneally, intramuscularly or subcutaneously, or administered by infusion techniques. For such parenteral administration the active agent is best used in the form of a sterile aqueous solution which may contain other substances, for example, enough salts or glucose to make the solution isotonic with blood. The aqueous solution should be suitably buffered (preferably to a pH of from 3 to 9), if necessary. The preparation of suitable sterile parenteral formulations is readily accomplished by terminal sterilisation methodology or by aseptic manufacture using standard pharmaceutical techniques well known to those skilled in the art.

Thus unit doses of the active agent may contain from 0.001 mg to 20g of active agent for administration singly or two or more at a time, as appropriate. For example acarbose has been administered at 15g per animal per day in 2 separate feeds. A target range for an active compound is up to ca. 3g/animal/day. The vet/farmer in any event will determine the actual dosage that will be most suitable for any individual animal or group of animals and it may vary

with the age, weight, diet and response of the particular animal. The above dosages are exemplary of the average case. There can, of course, be individual instances where higher or lower dosage ranges are merited and such are within the scope of this invention. The skilled person will appreciate that, in the treatment of certain conditions such as acute acidosis the

5   active agent may be given as a single dose as needed or desired.

The active agent will normally be administered orally or by any other suitable route (which can eventually reach the rumen), in the form of preparations comprising the active ingredient, optionally in the form of a non-toxic organic, or inorganic, acid, or base, addition salt, in an acceptable veterinary/pharmaceutical dosage form. Depending upon the disorder

10   and animal to be treated, as well as the route of administration, the compositions may be administered at varying doses (see below).

While it is possible to administer the active agent directly without any formulation, the active agents are preferably employed in the form of a pharmaceutical, or veterinary, formulation comprising a pharmaceutically, or veterinarily, acceptable carrier, diluent or

15   excipient and active agent. The carrier, diluent or excipient may be selected with due regard to the intended route of administration and standard pharmaceutical, and/or veterinary, practice. Compositions comprising the active agent may contain from 0.1 percent by weight to 90.0 percent by weight of the active ingredient.

The formulations will vary with regard to the weight of active compound contained

20   therein, depending on the species of animal to be treated, the severity and type of condition and the body weight of the animal. For parenteral and oral administration, typical dose ranges of the active ingredient are 0.0001 to 1000 mg per kg of body weight of the animal. Preferably the range is 0.001 to 20 mg per kg. For example acarbose was administered at 16mg/kg. More preferably the range is 0.001 to 5 mg/kg, and most preferably 0.001 to 0.5
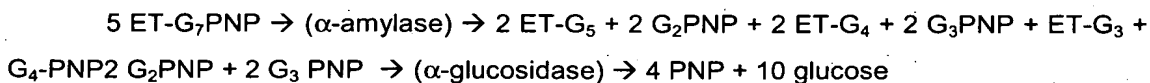
25   mg/kg.

It is to be appreciated that all references herein to treatment include curative, palliative and prophylactic treatment.

The efficacy of agents can be demonstrated using the following Test Methods, in which acarbose is used as an example of a suitable amylase and/or α-glucosidase inhibitor.

30   <div align="center">Test Methods</div>

Rumen bacterial amylase assay - protocol 1

The assay utilises a Sigma amylase kit (577) to determine whether compounds inhibit the action of rumen fluid supernatant amylases. The enzymatic reactions involved in the assay are as follows:

35    $5 \text{ ET-G}_7\text{PNP} \rightarrow (\alpha\text{-amylase}) \rightarrow 2 \text{ ET-G}_5 + 2 \text{ G}_2\text{PNP} + 2 \text{ ET-G}_4 + 2 \text{ G}_3\text{PNP} + \text{ET-G}_3 +$
$\text{G}_4\text{-PNP2 G}_2\text{PNP} + 2 \text{ G}_3 \text{ PNP} \rightarrow (\alpha\text{-glucosidase}) \rightarrow 4 \text{ PNP} + 10 \text{ glucose}$

α-Amylase hydrolyses 4,6-ethylidene-G_7-PNP (ET-G_7PNP) to G_2, G_3 and G_4 PNP fragments. α-Glucosidase (α-1,4-glucan glucohydrolase EC 3.2.1.3) hydrolyses G_2PNP and G_3PNP to yield p-nitrophenol and glucose. Five moles of substrate (ET-G_7PNP) is hydrolysed to yield 4 moles of p-nitrophenol. p-Nitrophenol absorbs light as 405nm, and following a two minute lag period the rate of increase in absorbance at 405nm is directly proportional to α-amylase activity in the well.

Rumen fluid was collected from 4-month-old Hereford x Friesian calves (125-135 kg, supplied by Cwmnant Calves Ltd. Cwmnant. Tregaron. Ceredigion) fed on diet GH 313. The rumen fluid was collected from slaughtered calves into pre-warmed vacuum flasks, as soon as possible after euthanasia. It was then filtered through a double layer of absorbent gauze (Absorbent gauze BP, GAUZ 4 from Robert Bailey plc, Stockport) to remove hay and feed particles. The liquid was centrifuged at a relative centrifugal force of 23,300 for 60 minutes, and the supernatant decanted, avoiding contamination from the loose top layer of the pellet by careful pouring. The supernatant was then aliquoted into 50ml plastic tubes and frozen at -20°C. When required for use in an assay the rumen fluid supernatant was thawed by standing the tubes in cold water. Test compounds or controls were dispensed into the 96-well assay plate at 4μl/well. 100μl per well of Sigma amylase reagent 577 (made up to half the volume described in the instructions (i.e. 10ml for a 577-20 vial)) was then added to each well, followed by 100μl per well of rumen fluid supernatant. A T=0 reading at 405nm was taken at this stage using an Anthos plate reader. The plate was then incubated at room temperature or 37°C until an optical density window of approximately 1.000U was seen (typically one hour at 37°C or three hours at room temperature). A second reading was taken at 405nm, and the first reading subtracted from it. Active compounds cause a reduction in the optical density readings when compared to the control without the agent being tested.

Results - IC50s in rumen fluid amylase screen (using Sigma kit 577)

Compound	Average IC50 (μM)	Number of assays
Acarbose	2.03	n=34
Trestatin A	0.17	n=8
Trestatin B	2.89	n=6
Trestatin C	0.09	n=6
V-1532	0.57	n=2
Example 7	2.06	n=8
Example 8	0.44	n=10

Dose response of acarbose in the rumen bacterial amylase assay [acarbose concentration in Molar units]

EC50 | 1.025e-006
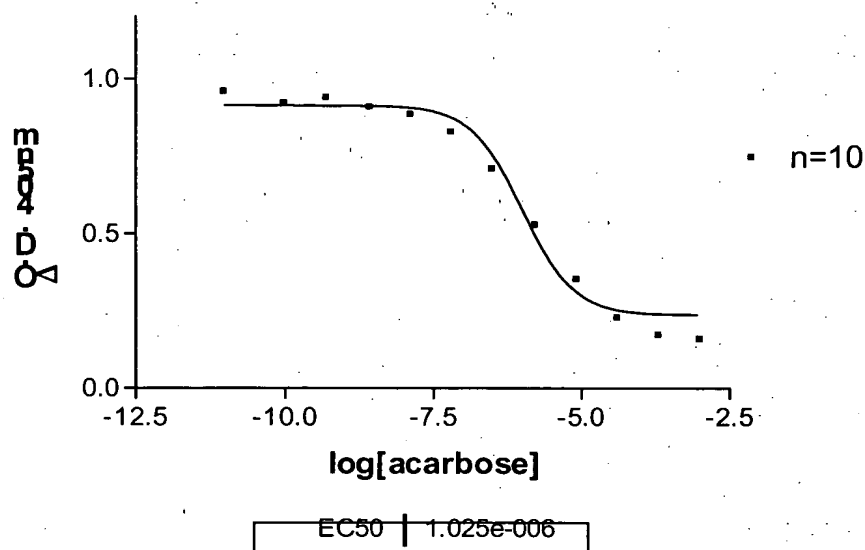
Rumen fluid glucosidase assay protocol

This assay is used to determine IC50 values for inhibitors of bacterial glucosidase activity from bovine rumen fluid cell suspension (RFCS) using a colorimetric assay.

5   The assay measures conversion of maltose into glucose. Rumen fluid cells are incubated with maltose in the presence of inhibitors, and the amount of glucose produced is assessed using a red colourimetric endpoint. The higher the level of inhibition the lower the glucose produced and the less red colour produced. The plates are read at 450nm.

Main reaction:

10   •   Maltose + glucosidase → glucose

Glucose assay

•   Glucose + ATP → G-6-P and ADP (Hexokinase and Mg^{2+})

•   G-6-P and NADP → 6-phosphogluconate (6-PG) and NADPH

•   NADPH + phenazine methosulfate (PMS) → NADP + PMSH

15   •   PMSH + INT (iodonitrotetrazolium chloride) → PMS + INTH

INTH is deep red coloured.

Rumen fluid was collected from a fistulated five year old dry Guernsey donor cow fed twice daily on 1.4kg GH313 and 2.3kg hay. The rumen fluid was collected into pre-warmed vacuum flasks. It was then filtered through a double layer of absorbent gauze (Absorbent

20   gauze BP, GAUZ 4 from Robert Bailey plc, Stockport) to remove hay and feed particles. The liquid was centrifuged at a relative centrifugal force of 650 for 15 minutes to remove food particles and protozoa. The supernatant was decanted into fresh tubes and centrifuged at a relative centrifugal force of 23,300 for 60 minutes. The supernatant and the loose top layer of the pellet were discarded. The pellet was resuspended in PBS at 1:8 of original volume i.e.

25   50ml of PBS for the pellet from 400ml of rumen fluid, and frozen at -20°C. When required for

use the cells were thawed by standing the tube in cold water, and then diluted to 4.5μl of cells/well (45μl/ml).

Test compounds or controls were dispensed into the 96-well assay plate at 2μl/well, followed by 50μl per well of 10mM maltose and 50μl/well of rumen fluid cell suspension. The
5   plate was incubated at 37°C for 1 hour. Sigma glucose detection kit 115A was reconstituted by addition of 17ml of Millipore water and 4ml of colour reagent to each vial. 100μl of this solution was added per well and the plate returned to the 37°C incubator for 45 minutes. The plate was then read at 450nm. Active compounds cause a reduction in the optical density readings when compared to the no-inhibitor control wells.

10   Results - IC$_{50}$s in rumen fluid glucosidase screen

Compound	Average IC50 (μM)	Number of assays
Acarbose	1.08	n=7
Trestatin A	33.6	n=3
Trestatin B	4.10	n=2
Trestatin C	148.5	n=2
V-1532	65.5	n=2
Example 7	6.38	n=1
Example 8	13.1	n=2

Rumen bacterial amylase assay protocol 2

The assay utilises digestion of amylose covalently linked to Remazol Brilliant Blue R to determine whether compounds inhibit the action of rumen fluid supernatant amylases.
15   When the insoluble substrate is incubated with amylase blue dye is released into the well. This can be measured spectrophotometrically to determine how much amylase activity is present, and whether test compounds are inhibitors of amylase.

Rumen fluid was collected from a fistulated five year old dry Guernsey donor cow fed twice daily on 1.4kg GH313 and 2.3kg hay. The rumen fluid was collected into pre-warmed
20   vacuum flasks. It was then filtered through a double layer of absorbent gauze (Absorbent gauze BP, GAUZ 4 from Robert Bailey plc, Stockport) to remove hay and feed particles. The liquid was centrifuged at a relative centrifugal force of 23,300 for 60 minutes, and the supernatant decanted, avoiding contamination from the loose top layer of the pellet by careful pouring. The supernatant was then aliquoted into 50ml plastic tubes and frozen at -20°C.
25   When required for use in an assay the rumen fluid supernatant was thawed by standing the tubes in cold water. 100μl per well of a 2% suspension of amylose azure (Sigma A3508) was added to each well from a beaker that was stirred throughout to ensure an even distribution of substrate. Test compounds or controls were dispensed into the 96-well assay plate at

4μl/well, followed by 100μl per well of rumen fluid supernatant. The plate was then incubated at 37°C for 2.25 hours. 100μl of liquid was removed gently from each well using a 12-channel pipette, transferred to a fresh 96-well plate and read at 620nm. Active compounds cause a reduction in the optical density readings when compared to the no-inhibitor control wells.

5   Results - IC50s in rumen fluid amylase screen (amylose azure)

Compound	Average IC50 (μM)	Number of assays
Acarbose	2.39	n=4
Trestatin A	0.79	n=2
V-1532	2.07	n=2
Example 7	0.56	n=2
Example 8	9.45	n=2

Protocol for determination of minimum inhibitory concentrations (MICs) in Aerobes

MICs were determined by a standard agar dilution technique according to the National Committee for Clinical Laboratory Standards (NCCLS, M7 Edition A2). An outline of

10   the method employed is detailed below.

The MICs were determined using the standard test medium, Mueller Hinton (MH) agar (Unipath).

Preparation of agar plates: 19ml of test medium was added to appropriate doubling dilutions of test compound (1ml) and mixed thoroughly. The mixture was poured into a petri

15   dish (90mm) and the agar allowed to solidify.

Preparation of inoculum: Four to five colonies of the test organism were inoculated from a MH agar plate culture into 10ml MH broth (Unipath). The broth was incubated at 37°C until visibly turbid. The density of the culture was adjusted to a turbidity equivalent to that of a 0.5 McFarland standard by the addition of saline (0.85% v/v).

20   Inoculation of agar plates: The plates were dried for approximately 1 hour in a 37°C incubator. Plates were inoculated with a Multipoint Inoculator (Denley). The pins on this device deliver 0.001ml inoculum to the plate (equivalent to 10^4-10^5 organisms).

Incubation of plates: Plates were inverted and incubated at 37°C for 18 hours.

Determination of endpoints: MICs were recorded as the lowest concentration of test

25   compound that completely inhibited growth, disregarding a single colony or a faint haze caused by the inoculum.

References for this section:

National Committee for Clinical Laboratory Standards

Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically

30   – second edition.

Approved standard reference methods for the determination of MIC of aerobic bacteria by broth macrodilution, broth microdilution and agar dilution. Chair holder J. Allan Waitz, PhD DNAX Research Institute, NCCLS Document M7-A2

Villanova, Pa.: NCCLS, 1990

5   RESULTS USING ACARBOSE IN THIS TEST:

No.	Bacterial species	MIC (µg/ml)
1	E. coli ATCC 10418	>128
2	E448	>128
3	E454	>128
4	E459	>128
5	E450	>128
6	E476	>128
7	E461	>128
8	E516	>128
9	E517	>128
10	E520	>128
11	Salm. enteritidis B1234	>128
12	B1227	>128
13	B1240	>128
14	B1218	>128
15	B1231	>128
16	B1233	>128
17	B1232	>128
18	B1235	>128
19	E. faecium 1.1.7	>128
20	1.2.4	>128
21	1.1.6	>128
22	28.7.7	>128
23	1.2.6	>128
24	28.6.7	>128
25	5.4	>128
26	4.5	>128
27	3.1	>128
28	10.1	>128
29	E. faecalis 1.3.10	>128

No.	Bacterial species	MIC (µg/ml)
30	*1.4.12*	>128
31	*1.1.16*	>128
32	*1.6.6*	>128
33	*1.3.13*	>128
34	*1.10.4*	>128
35	*28.5.7*	>128
36	*1.9.5*	>128
37	*1.1.4*	>128
38	*28.6.9*	>128
39	*S. aureus 3.3*	>128
40	*3.4*	>128
41	*3.5*	>128
42	*5.1*	>128
43	*6.1*	>128
44	*8.2*	>128
45	*8.3*	>128
46	*9.3*	>128
47	*10.3*	>128
48	*10.4*	>128
49	*NCTC 6571*	>128

DIETS USED IN THE FOLLOWING TESTS

[All diets were provided by Grain Harvesters Ltd, The Old Colliery, Wingham, Canterbury, Kent CT3 1LS, England.]

GH313:

Material	Inclusion	Analysis	
BARLEY (fine)	24.000	VOLUME	100.000
WHEAT	10.000	PROTEIN	14.005
WHEAT MIDDLINGS	11.900	OIL	3.794
SUNFLOWER MEAL (EXT)	5.100	FIBRE	8.501
RAPESEED MEAL (EXT)	10.000	STARCH	27.309
PEAS	7.500	STARCH + SUGAR	32.783
WHOLE LINSEED	1.200		

GRAIN SCREENINGS	7.500
UNMOLASSED SUGAR BEET	13.900
LIMESTONE GRANULES	1.300
SALT	0.800
GHS CATTLE SUPP.	0.250
ADDAROME Cattle Supplement	0.020
MOLASSES	5.000
VEGETABLE FAT (MIXER)	1.500

<u>99.970</u>

GH633

Material	Inclusion	Analysis	
BARLEY (fine)	22.100	VOLUME	100.000
WHEAT MIDDLINGS	17.500	PROTEIN	15.122
MAIZE GLUTEN	8.800	OIL	4.700
FISHMEAL (PROVIMI 66)	2.500	FIBRE	9.601
SUNFLOWER MEAL (EXT)	4.500	STARCH	17.175
RAPESEED MEAL (EXT 00)	5.000	SUGAR	8.770
LUCERNE PELLETS	10.000		
MOLASSED SUGARBEET	20.000		
LIMESTONE FLOUR	0.400		
SALT	0.650		
INT LAMB SUPPLEMENT (10 kg)	1.000		
SPRAY VEGETABLE FAT	1.600		
MOLASSES	5.000		
MIXER VEGETABLE FAT	1.000		

<u>100.050</u>

GH651:

Material	Inclusion	Analysis	
BARLEY (fine)	15.000	VOLUME	100.000

WHEAT	50.000	PROTEIN	13.984
WHEAT MIDDLINGS	11.000	OIL	3.206
RAPESEED MEAL (EXT)	14.400	FIBRE	4.539
LIMESTONE GRANULES	1.900	STARCH	39.523
DICALCIUM PHOSPHATE	0.030	STARCH + SUGAR	44.838
SALT	0.820		
GHS CATTLE SUPPLEMENT	0.250		
COLBORN No. 3	0.100		
MOLASSES	5.000		
VEG FAT (MIXER)	1.500		

<u>100.000</u>

GH654:

Material	Inclusion	Analysis	
WHEAT MIDDLINGS	22.000	VOLUME	100.000
MAIZE GLUTEN	16.600	PROTEIN	12.867
SUNFLOWER MEAL (EXT)	7.000	OIL	4.688
RAPESEED MEAL (EXT)	4.200	FIBRE	13.991
OATFEED	10.000	STARCH	10.109
N. I. Straw	10.000	SUGAR	5.352
UNMOLASSED SUGAR BEET	20.000		
LIMESTONE GRANULES	1.200		
SALT	0.680		
CALCINED MAGNESITE	0.540		
AMMONIUM CHLORIDE	0.050		
COLBORN Cattle Supplement	0.250		
ADDAROME Cattle Supplement	0.040		
MOLASSES	5.000		
VEG. FAT (MIXER)	2.500		

<u>100.060</u>

<u>Evaluation of agents using the rumen simulation technique (RUSITEC) to model chronic acidosis.</u>

The in vitro rumen simulation technique (RUSITEC), first described by Czerkawski and Breckenridge (1977) was used to evaluate the effect of the bacterial α-amylase and/or α-glucosidase inhibitor acarbose on daily pH profiles and VFA production using a commercial cattle concentrate ration (GH313 - see later). Feeding 30g/d of this ration with 2.5g/d chopped

5   barley straw had previously been found to give total volatile fatty acid (VFA) concentrations of more than 150mM i.e. concentrations associated with chronic acidosis in vivo (Nagaraja, Galyean & Cole, 1998, supra)

Equipment: The apparatus consisted of two RUSITEC units each containing four-fermentation vessels. Each vessel had a volume of 1 litre, and was heated to 39°C in a water

10   bath. The feed was placed in a nylon bag (14 x 9 cm, 50 µm), and was gently agitated using a piston mechanism (8 strokes/min). Buffer (McDougall, 1948) was continuously infused at a rate of approximately 750ml/day by an eight channel peristaltic pump (Watson Marlow). The effluent was collected in 1litre glass bottles containing 20ml of oxalic acid solution (12g/100ml in deionised water). This was added to inhibit further microbial activity

15   Feed: Each vessel was fed daily with a bag containing 30g of the commercial pelleted ration GH313 (89% dry matter) and 2.5g barley straw (90% dry matter) chopped into 1-2cm lengths. 7g of corn starch (Sigma Cat. N°. S4126) was added to the liquid phase of all fermenter vessels at feeding on the last four days of the experiment to simulate acute acidosis.

20   Rumen fluid donor: Rumen fluid was collected from a five year old dry Guernsey cow. The animal was fed twice daily with 1.4kg GH313 and 2.3kg hay. Rumen contents were collected via a rumen fistula (Bar Diamond Inc. P.O.Box.60. Bar Diamond Lane. Parma. Idaho. 83660-0060. U.S.A)

Vessel inoculation: Rumen contents were taken from the fistulated donor animal at

25   08:00h (before the morning feed). The material was carried to the laboratory in pre-warmed insulated flasks, and then strained through four layers of cotton gauze into another pre-warmed insulated flask. 70g of the solid residue were weighed into each of eight nylon bags. One bag containing rumen solids, and one bag containing fresh feed was placed in the feed chamber for each vessel. The liquid contents of each vessel were 100ml deionised water,

30   200ml of buffer artificial and 500ml of rumen fluid. After assembling and sealing the vessels, they were placed in the water baths and the piston rod attached to the drive bar. The effluent tubes were placed in the collection flasks. The head space in each vessel was flushed with CO_2 for 2min., then the piston drive motor and buffer infusion pump were started.

Daily maintenance and sampling procedure: These procedures were carried out at

35   the same time each day. Eight feed bags were prepared, and a 1l dispenser bottle containing infusion buffer warmed to 39°C.

1.   Drive motors were switched off and the infusion pump stopped. Infusion lines clamped and disconnected from the pump.

2.   The fermentation vessels were removed from the water bath and serviced in turn.

5   3.   For each vessel, the feed chamber was extracted and feed bags exchanged. On Day 2 the new bag replaced the one containing rumen solids, whilst on subsequent days the new bag replaced the one that has been incubated for 48 hours. Chamber then replaced in fermentation vessel.

4.   The removed bag was placed in a small plastic bag and 25ml buffer added

10   from the dispenser. The bag was washed by squeezing in the buffer for 20 seconds, then the liquid was poured into the vessel. This washing procedure was repeated twice with fresh buffer.

5.   After reassembling the vessel, it was replaced in the water bath and attached to the drive bar. The buffer line was reconnected and the pH electrode relocated. The

15   effluent collection bottle was exchanged and the vessel headspace purged with CO_2 whilst the next vessel was being serviced.

6.   This process was repeated for all vessels, then the drive motors and infusion pump were restarted when gassing was complete. For the last vessel gassing was for a similar duration as for the other vessels.

20   Treatment with acarbose: Acarbose was obtained as Glucobay™ tablets (Bayer, AAH Pharmaceuticals) Each tablet contains 100mg acarbose. Duplicate vessels were treated with 0, 1, 10 and 100mg/d of acarbose by adding 1 tablet to each of two feed bags to give 100mg/vessel/d. The lower doses were prepared by dissolving/suspending a tablet in 10ml buffer (giving a 10mg/ml solution of acarbose). One ml of this solution was then added to 9ml

25   of buffer, giving a 1mg/ml solution. One ml of each solution was then added to the contents of two feed bags, to give 10 and 1mg/vessel/d. Finally, the acarbose was dried on to the feed by leaving the bags at room temperature overnight.

Analyses: Dry matter losses from the nylon bags after 48 hours incubation were measured by drying the washed bag contents in an oven for 23h at 65°C. Effluent samples

30   (10ml) were taken daily and stored at -20°C for subsequent VFA and lactate analysis. pH was automatically recorded at 17min. intervals using equipment supplied by Philip Harris Education. A combination electrode was fitted in each vessel via a gas-tight port in the lid. Each electrode was connected to a SensorMeter, and four SensorMeters were connected to one DL plus 128 datalogger. The recorded pH values were downloaded to a PC running

35   Datadisk 32 software (Philip Harris Education) and then transferred to a spreadsheet for further analysis. The electrodes were removed from the vessels (when the feed bags were being changed), rinsed and placed in pH7.0 standard buffer. The reading were 7.0+/- 0.1units

throughout the experiment. The electrodes were recalibrated to pH 7.0 before being replaced in the vessels. VFAs were measured by adding 0.1ml of a solution containing a mixture of 25g/100ml metaphosphoric acid and 1.2g/100ml crotonic acid to 1ml of effluent. This mixture was centrifuged for 10min. at 12000g and an aliquot of the supernatant transferred to an autosampler vial. VFAs were resolved and quantified on a Hewlett Packard 6890 series gas chromatograph fitted with an autosampler and flame ionisation detector. The acids were resolved on a SGE Ltd 25metre BP21 column (0.33mm O.D., 0.22mm I.D. 0.25um film thickness). Nitrogen was used as the carrier gas with a flow rate of 1.9ml/min. The oven temperature was 165°C, and injection ports and detectors were held at 250°C. Concentrations were calculated by using crotonic acid as an internal standard, and the system was calibrated using a standard solution containing acetic, propionic, butyric, iso-valeric and n-valeric acids. L-lactic acid was measured using Sigma kit 826, and D-lactate was measured using the same procedure, except L-LDH was replaced with D-LDH (Sigma Catalogue N° L2011) and L-lactate was replaced with D-lactate (Sigma Catalogue N° L0625). Assays was carried out on a 96-well microtitre plate and the absorbances measured using an Anthos microtitre plate reader fitted with a 340nm filter. The system was calibrated by preparing solutions of D- and L-lactate from 0 to 100mM.

Schedule:

Day

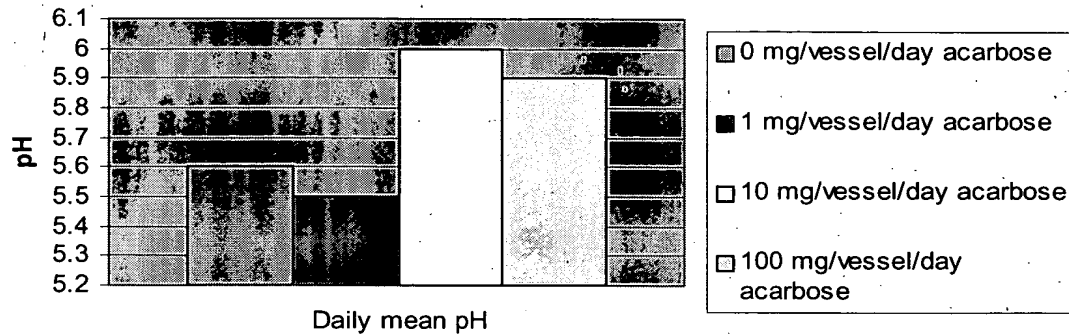| 0 | Inoculate. |
3	Start collecting effluent
5	Start daily pH measurement.
10	Begin dosing with acarbose (0,1,10 or100mg/vessel/d) to pairs of vessels. Continue to end of experiment.
18	Add extra starch to all vessels.
22	End experiment.

References for this section:

Czerkawski, J.W. and Breckenridge, G. 1977. Design and development of a long-term rumen simulation technique (RUSITEC). *British Journal of Nutrition*, **38**, 371-384.

McDougall, E.I. 1948. Studies on ruminant saliva. 1. The composition and output of sheep's saliva. *Biochemical Journal*, **43**, 99-109.

Rusitec results: acarbose

Daily mean pH in Rusitec chronic acidosis model treated with acarbose

Daily mean pH

- 0 mg/vessel/day acarbose
- 1 mg/vessel/day acarbose
- 10 mg/vessel/day acarbose
- 100 mg/vessel/day acarbose

Comparing the treatment period with the preceding control period indicated a dose-related change in VFA production of -16%, -10% +3% and -3% in response to additions of 100, 10, 1 and 0mg/vessel/d of acarbose. There was a general shift of fermentation products from acetate and propionate to butyrate with all treatments between the control and treatment periods, resulting in increases in butyrate production of 134%, 76%, 27% and 24% respectively for the above doses. There was no L-lactate accumulation in this study, confirming that the model represented chronic rather than acute acidosis.

RUSITEC Result using Example 8 and Acarbose

Experimental outline:

Daily maintenance procedures as previously described for Acarbose.

Rumen fluid donor cow- Fed GH313 pellets plus barley straw.

Daily Feed- 30g GH313 pellets plus 2.5g chopped barley straw.

Treatment Preparation

Acarbose: 1 tablet added to each bag to give 100mg/vessel/d

Example 8: Eight x 57mg pre-weighed samples stored in fridge in 380 2.1. On the day before the feed bags were to be placed in RUSITEC, a 57mg sample was dissolved in 2.28ml buffer (25mg/ml). 0.21ml of this solution was added to 1.9ml buffer to give a 2.5mg/ml solution. 1ml of each solution was added to a prepared feed bag to give 25 and 2.5mg/vessel/d. and dried overnight at room temperature.
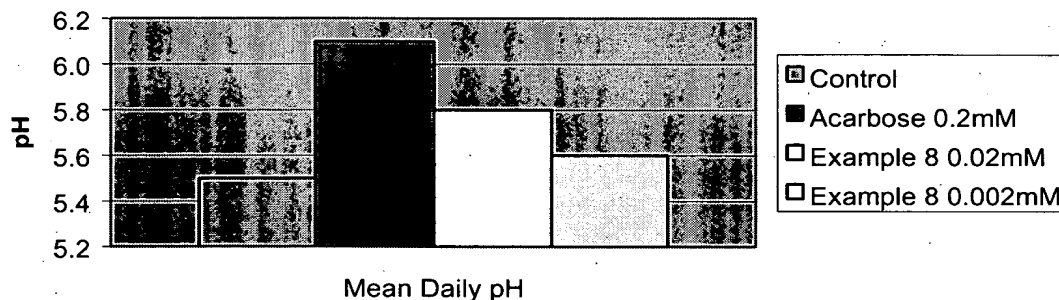
Schedule:

Day

0   Inoculate.

4   Start collecting effluent

5   Start daily pH measurement

9   Review data, allocate vessels to treatments.

10   Begin treatments.

18   End experiment.

Effect of treatment with acarbose or Example 8 on mean daily pH in the Rusitec chronic acidosis model

Mean Daily pH

Results and Conclusions: In this experiment 0.02mM Example 8 gave an increase in mean daily pH of 0.3 units compared to 0.7 units for 0.2mM acarbose. Treatments of 100mg acarbose, 25 or 2.5mg Example 8 per vessel per day, or none (control) caused changes in total VFA production of -15%, -8%, -1% and -11% when compared with the preceding control period. There was a trend for redistribution of fermentation products, with butyrate production increasing in the treatment period compared with the control. The proportional increases were 113%, 20%, 18% and 1% respectively. There was no L-lactate accumulation in this study, confirming that the model represented chronic rather than acute acidosis.

IN VITRO RUMEN PROPIONIC ACID SCREEN

REAGENTS

Rumen Fluid: An eight year old dry Guernsey cow, fitted with a rumen fistula (Bar Diamond Inc. P.O.Box.60. Bar Diamond Lane. Parma. Idaho. 83660-0060. U.S.A) was fed twice daily with 1.4kg GH633 and 2.3kg hay. This animal was used as a source of rumen contents, which were taken at 08:00h (before the morning feed). The material was carried to the laboratory in a pre-warmed insulated flask, and then strained through four layers of cotton gauze into another pre-warmed bottle, which was stored in an incubator at 40°C until the fluid was dispensed into the assay tubes.

Buffer: Dissolve the following in deionised water.

	g/l	g/500ml
$Na_2HPO_4.2H_2O$	9.88	4.94
KH_2PO_4	3.40	1.70
$NaH_2PO_4.H_2O$	1.11	0.55

Adjust to pH 7.0 with 1M NaOH.

Deoxygenate by bubbling with an oxygen-free gas mixture (10%CO_2, 5%H_2 in nitrogen) for at least 5min.

Substrate mixture:

68g corn starch. Ex Sigma Cat. S-4126.

17g α-cellulose. Ex Sigma Cat. C-6429.

15g Type 1 soya flour. Ex Sigma Cat. S-9633.

Mix well.

Immediately before use, suspend in buffer at 200mg/ml.

Metaphosphoric/crotonic acids solution:

The following were dissolved in deionised water.

25% (w/v) metaphosphoric acid. Ex. BDH Cat. 291904A plus

1.2% (w/v) crotonic acid. Ex Sigma Cat. C-4630

VFA standard mixture:

The following were dissolved in 100ml of deionised water.

	mwt	nominal weight (mg)	concentration mM
Sodium acetate. Ex Sigma Cat. S-7670	136.1	680	50
Sodium propionate. Ex Sigma Cat. P-1880	96.1	192	20
Sodium butyrate. Ex Sigma Cat. B-5887	110.1	110	10
n-Valeric acid. Ex Aldrich Cat. 24,037-0	102.1	102	10
iso-valeric acid. Ex Sigma Cat. I-7128	102.1	102	10

1ml of metaphosphoric/crotonic acids solution added to 10ml of VFA mixture then aliquoted into automatic liquid sampler vials.

Procedure:

The assay was conducted in 16ml Sorvall centrifuge tubes

A tablet containing 100mg of acarbose was placed in 10ml of buffer and shaken until the tablet was completely disrupted, giving a 10mg/ml solution of acarbose. This solution was serially diluted to 2, 0.4, 0.08 and 0.016mg/ml with buffer.

One ml of these solutions was added to triplicate assay tubes (giving final assay mixture concentrations of 1000, 200, 40, 8 and 1.6ug/ml). Control tubes were prepared by replacing the acarbose solution with buffer. One ml of substrate suspension was added to all the assay tubes, followed by 3ml of warmed degassed buffer and then by 5ml of strained rumen liquor. Suba-Seal stoppers (No. 29) were fitted, and the head pressure in the tubes reduced by passing a hypodermic needle attached to a vacuum line through the stopper until the tube contents frothed. The tubes were then placed in a 40°C incubator for 6 hours and shaken hourly.

Pre-incubation VFA concentration were determined by preparing three tubes as for incubation but 1ml metaphosphoric/crotonic acids solution was added immediately following the rumen liquor. These tubes were stored at 4°C and processed with the post-incubation tubes.

5 The incubation was terminated after 6h by removing the stoppers and adding 1ml of metaphosphoric/crotonic acids solution. The tubes were then centrifuged for 8minutes at 18,000g at 4°C, and an aliquot of the supernatant stored in an automatic liquid sampler vial until required for VFA analysis by gas chromatography. (as described in RUSITEC protocol)

RESULT CALCULATION:

10 Production of total VFA and propionate during the incubation was determined as follows. Firstly, first the pre-incubation concentrations of total VFA and propionic acid were calculated as the mean of the analyses of the pre-incubation samples. Then for each incubated sample, post- minus pre-incubation concentration gave production during incubation. The molar proportion of propionic acid in total VFA produced during the incubation 15 was also calculated.

The total VFA and %propionic acid values were meaned for the replicate tubes, and the mean control total VFA and %propionic acid values normalised as 100%, then the change caused by the test treatments expressed relative to this value.

acarbose		
*Dose	Total VFA	% Propionate
µg/ml		
1000	50	88
200	58	81
40	63	74
8	72	74
1.6	92	94
0.32	96	97
0	100	100

20 *In vivo* testing in fistulated cattle

Objective: To determine the effect of the agent for testing, in this case acarbose, on chronic rumen acidosis induced in fistulated cattle. A rumen pH profile representative of chronic acidosis was induced by stepwise increase in the level of concentrate feeding of a specified diet and a reduction in the roughage offered. This was followed by treatment of each 25 animal with acarbose, administered *via* a permanent rumen fistula, to assess its ability to normalise rumen pH.

Experimental Animals: Six fistulated Hereford x Friesian steers, weighing 170-230kgs (supplied by Cwmnant Calves Ltd. Cwmnant, Tregaron, Ceredigion)

Treatment: Glucobay® 100. Acarbose 100mgs per tablet.

5   Management: The cattle were fed GH651 cattle high cereal beef pellets (variable amount) with barley straw (variable amount) divided over two equal feeds, given at around 08.00hr and 14.30hr each day. Precise feeding times were recorded. Water was available *ad-lib*. Cattle were individually housed in pens (9 square metres per pen) in a building environmentally controlled to 16°C.

Design:

Group	Treatment	Form	Route	Acarbose (g/trt)	Volume (ml)	No. of Animals
1	Acarbose	Aq. Sol.	Through fistula	4.0	100	6

10

While chronic acidosis was being induced, manual pH measurements were taken approximately 5 and 8 hours post-morning feed. Rumen fluid samples were taken for VFA and lactate analysis. Once a suitable pH profile was generated (see procedure section), each steer was fitted with a harness to carry an automated pH sampling and recording device

15   (Philip Harris Plus 128 Data logger. + p.H. First Sense Recorder). Rumen pH values were automatically recorded every 17 minutes for a maximum of 21 days. Rumen fluid samples (10ml) were taken twice daily for measurement of VFA levels, molar ratios and lactate levels. These were collected (by manually removing a sample of rumen content with a small stainless steel ladle, filtering and transferring to a 10ml polypropylene vial) just before

20   acarbose was added to the rumen at both dosing times. The pH probes were removed for cleaning and recalibration immediately pre-morning feed.

Results: The daily pH curves were used to calculate the period that rumen pH was below pH5.5, and therefore indicative of chronic acidosis.

Time below pH 5.5 during control and acarbose treatment phases
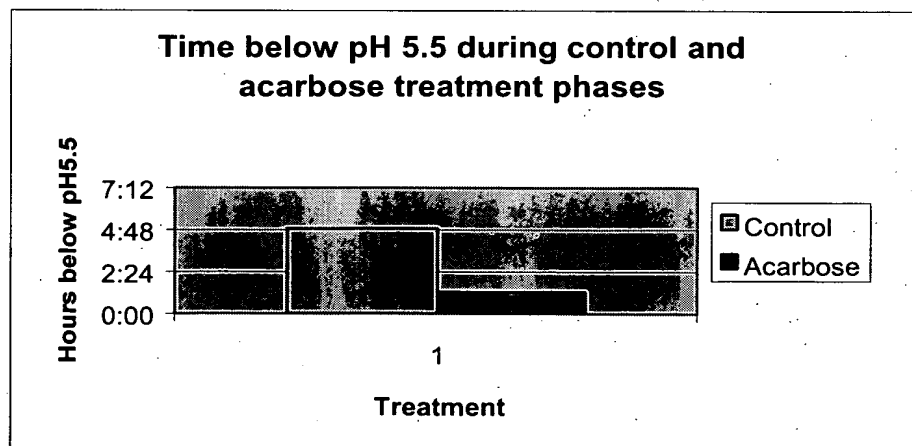
Rumen fluid samples were taken at 13:00 and 16:00 i.e. before and after the afternoon feed. There was little difference in total VFA concentrations at the 13:00 samples, but the VFA concentration in the 14:00 samples fell during the treatment period. This was consistent with the pH profiles. At all sampling times the percentage of propionate was lower during the treatment period. There was no accumulation of lactic acid in the rumen fluid samples, indicating that the animals did not experience acute acidosis during the experiment.

EFFICACY OF ACARBOSE IN LACTATING DAIRY CATTLE

The study measured the effect of acarbose on rumen pH, milk yield and milk composition in lactating cows in which chronic acidosis had been induced by offering a highly fermentable diet. The study included measurement of rate of adaptation to the introduction and removal of acarbose.

Experimental animals were six lactating multiparous Holstein/Friesian cows between 5 and 11 years of age and 500-750kg, with permanent rumen cannulas. The animals started the experiment in early lactation, but not before peak lactation, to allow greater experimental sensitivity. The principal measurements in this study were pH in the ventral sac of the rumen, milk yield and milk composition.

Management practices complied with the UK Home Office code of practice for the Housing and Care of Animals Used in Scientific Procedures (1989).

DESIGN: Animals enrolled into the study received test article for 21 consecutive days from the start time points described below.

Treatment Group	Supplement fed from Day 0	Supplement fed from Day 21	Supplement fed from Day 42	Number of Animals
T01	A	B	A	3
T02	B	A	B	3

A= Control supplement ration

B= Acarbose containing supplement ration

PROCEDURE:

Masking/Bias-Reducing Methods: Six lactating multiparous Holstein/Friesian cows were enrolled on to the study on Day -1. Animals were paired based on their calving date (cows with similar dates paired together). Within each pair, one animal received T01 and the other T02. The treatment was assigned at random. Where possible, the randomisation was constrained so that the average feed intakes per treatment group were similar.

Methods: At approximately two weeks prior to Day 0, eight animals began a preliminary feeding period designed to identify a feeding regimen that induced acidotic pH levels in the rumen. Each cow was held in a tie stall from this point until completion of the study. Control total mixed ration (TMR) was fed and where necessary the amount and

composition adjusted to establish a minimum rumen pH of 5.0-5.5. The mean intake over the last 5 days of the preliminary period was calculated and this mean amount was offered to each animal throughout the trial. Unconsumed food was removed and weighed on a daily basis and prior to the morning feed.

The TMR was supplemented with 0.5kg/day ground wheat with either no additive (Control) or containing the test article (acarbose at 15g per day), and offered separately in equal halves over the morning and afternoon feeds. An automatic watering system was also used to offer water *ad-libitum*.

TMR composition:

Ingredient	% in total ration DM
Grass silage	10.0
Maize silage	30.0
Cracked wheat	16.7
Ground barley	9.2
Rapeseed meal	4.1
Soyabean meal	6.1
Molassed SBP	9.2
Wheatfeed	8.2
Regumaize	4.0
Fishmeal	1.0
Minerals	1.5
Total	100.0

From the start of the study, all animals were milked twice daily through a pipeline system at approximately 06.30h and 16.00h. Milk yield weight was manually recorded and then transcribed to an electronic daily milk file.

On Day -1, each animal was physically examined by a veterinary surgeon to assess physical and clinical normality. Animals met all of the inclusion criteria and none of the exclusion criteria.

On Day 0, the test article feeding regimen described in DESIGN began. Three animals followed the design Control/Treatment/Control (A/B/A) on three consecutive experimental periods whilst a second group of three animals followed the opposite treatment sequence (B/A/B). Experimental feeding periods lasted 3 weeks, consisting of 2 weeks for adaptation and a final week for detailed measurements.

Animals were fed twice daily at unequal intervals at approximately 08.00h and 15.00h (i.e. 7 and 17-h intervals) to allow rumen sampling for pH measurement to be concentrated during the period of minimum pH (estimated to be 2-4 h after the second feed). Each animal

was offered 0.5kg/day ground wheat supplement to the TMR divided equally over the two feeds and containing either no additive (Supplement A) or the test article (Supplement B).

Each animal completed the study after the final pH measurement of the third experimental period on Day 62.

5   MEASUREMENTS: Automated pH measuring equipment (Philip Harris Plus 128 Data logger + pH First Sense Recorder) was used to record rumen pH values every 17 minutes. A 10mL-15mL sample of rumen fluid taken at 07.30hrs and two samples taken at approximately minimum pH (at approximately 18.00hrs and 20.00hrs) were frozen immediately at -20°C for further analysis. Fresh weight of TMR offered and refused was recorded daily for each
10   animal. During non-measurement weeks (i.e. prelim period and adaptation weeks 1 and 2 in each experimental period), dry matter (DM) of main forage components (grass and maize silages) were determined approximately weekly (or more frequently if it appears necessary from visual assessment of the silage). For measurement weeks (i.e. week 3 in each experimental period), the DM of the TMR were measured daily for the last 5 days while a bulk
15   of each of the individual TMR components (grass silage, maize silage, concentrate mix, ground wheat) were prepared from the same 5 days for subsequent diet analysis (i.e. one sample of each main feed per period). Only one sample of Regumaize was taken during the study as it is a single bulk liquid. Refusals were sampled for DM determination during the last 5 days of each experimental period. Single 20ml milk samples for fat, protein and lactose
20   were taken at am and pm milkings on 3 alternate days in each adaptation week. During measurement week milk samples were taken on the last 5 consecutive days. Each milk sample was analysed separately. Additionally a further 20ml milk sample was taken on each occasion that milk was sampled during the measurement weeks and immediately frozen at approximately -20°C for possible subsequent analysis. Daily milk yield data was generated by
25   totalling the morning and afternoon milkings on a given date. Live weight was measured in each experimental period for each animal.

RESULTS: Rumen pH was calculated as time below pH5.5 (i.e. in a state of chronic acidosis) for the treatment and control periods. The average time below pH 5.5 was 4.3 hours for control periods and 3.4 hours for treatment periods. Milk fat increased dramatically in
30   treated animals, from an average of 1033 grams per day to 1281 grams per day. The proportion of fat in the milk was also increased, from 32.8g/L to 46.1g/L.

Acute acidosis model: assessment of acarbose

EXPERIMENTAL DESIGN:

Treatment		Route	No. Animals
No.	Description		
1	Control: ~200 mL water BID for 7 days pre-challenge, 12.5 g/kg BW of challenge mixture*	Cannula	5 (3 dry cows and 2 heifers)
2	Acarbose: 1.07 mg acarbose/kg BW dissolved in ~200 mL water BID for 7 days pre-challenge, 12.5 g/kg BW of challenge mixture containing .02g/kg BW acarbose	Cannula	5 (2 dry cows and 3 heifers)

*48.4% cornstarch, 48.4% ground corn, 2.1% sodium caseinate, 1.1% urea (food
grade) suspended in approximately 5 gallons lukewarm water

Procedures: Ten Holstein dried off cows and heifers (initial weight 740 ± 27 (SE) kg,
range = 606-870 kg) were group-housed during the pre-treatment period and individually
housed during the experimental period. Animals were moved to a head-gate during sample
collection, treatment and first challenge. Subsequently, they were sampled and dosed in their
individual pens. Animals were offered approximately 5 kg alfalfa hay, 16 kg silage, 6 kg
concentrate and .5 kg straw daily in a total mixed ration offered in two feedings (60:40
concentrate:roughage diet). They were bedded on straw only during the pre-treatment
period. Water was provided ad libitum. Animals were adapted to the lactating ration for at
least 10 days before treatments were administered. From a group of 6 dry cows and 5 non-
lactating heifers, ten were selected based on previous exposure to challenge and general
health. Animals were paired by body weight and were randomly assigned to control and
acarbose treatment groups within pair, ensuring similar distribution of heifers and cows. On
days 0-6 of treatment, animals received 1.07 mg acarbose/kg dissolved in ~200 mL water
through the rumen cannula just before AM (07:30) and PM (16:00) feedings. Treatment 1
animals received an equivalent amount of water only. On days 7 and 8, each animal was
administered a challenge through the cannula. When pH reached ~ 4.5 and there was
evidence of L-lactate production, acute acidosis was considered to be induced. When an
animal experienced acute acidosis by these criteria, rumen contents were removed and the
rumen inoculated with rumen contents from a donor animal. Animals were weighed on days -
1 and 5 for calculation of acarbose dosing and challenge amounts. To measure rumen fluid
pH animals were fitted with a harness to hold an automatic pH data recording system. The
rumen pH was recorded every 10 min during the days of challenge until an animal
experienced acute acidosis. Rumen fluid samples were taken (~50 mL) from the rumen

cannula through a filtered sampling tube. Sampling times were just before each challenge, and 3, 6, 8, 10 and 12 hrs after each challenge. The pH was measured immediately. Samples for VFA and lactate analysis were prepared by adding 10ml of rumen fluid to 1ml of a solution containing a mixture of 25g/100ml metaphosphoric acid and 1.2g/100ml crotonic acid straight

5   after collection. In some cases the sample was filtered through gauze before pH measurement and acid treatment. This mixture was centrifuged for 10min at 12000g. One aliquot of the supernatant was removed for immediate lactate analysis, one frozen for subsequent lactate analysis using Sigma kit 826. A third aliquot was transferred to an auto-sampler vial for subsequent VFA analysis. An initial determination of L-lactate was made

10   during the study to establish acidotic status using Sigma kit 735. Subsequently D and L-lactate were re-measured for statistical analysis as described below. VFA's were measured on a Hewlett Packard 6890 series gas chromatograph fitted with an auto-sampler and flame ionisation detector. The acids were resolved on a SGE Ltd. 25metre BP21 column (0.33mm O.D., 0.22mm I.D. 0.25um film thickness). Nitrogen was used as the carrier gas with a flow

15   rate of 1.9ml/min. The oven temperature was 165°C, and injection ports and detectors were held at 250°C. Concentrations were calculated by using crotonic acid as an internal standard, and the system was calibrated using a standard solution containing acetic, propionic, butyric, iso-valeric and n-valeric acids.

RESULTS:

20   RESULTS:

pH: For the challenge pH data are presented as a calculation of the time that rumen pH was below a range of cut-offs. The first challenge did not induce acute acidosis, but after the second challenge, four of the control cattle had rumen pH values below 4.5. The short duration below pH 4.5 was due to their removal from the study at that point. Rumen pH

25   remained above 5.0 in all the treated cattle, indicating that acarbose prevented acute acidosis following carbohydrate challenge. The treatment effect of reducing the number of cows that became acidotic was shown to be significant ($P < 0.5$) by a simple contingency table analysis.

Lactates: There was no lactate detected after the first challenge, but levels increased to >50mM for four of five controls within 10 hrs of the challenge on the second and remained

30   at zero in all the acarbose-treated animals. The fifth control, which had pH of ~5.0, had a maximum level of lactate of 6mM. Mean D-, L- and total lactates from the second challenge are summarized in the table. All lactates were higher in the control than treatment group and there was a trend for treatment by time interaction (the differences became greater over time).

Group Mean D-Lactate, L-lactate and Total Lactates in Samples from Day 2 Challenges (mM)

Hours Post Second Challenge	D-Lactate		L-Lactate		Total-Lactate	
	Control	Acarbose	Control	Acarbose	Control	Acarbose
0	11.48	0.67	3.82	0.35	15.30	1.02
3	42.93	3.63	15.33	1.28	58.26	4.91
5	58.19	10.91	17.80	3.08	76.08	13.99
7	74.69	18.00	18.98	4.93	93.92	22.93
P Values						
Treatment		.04		.03		.04
Time		<.01		<.01		<.01
Treatment *Time		.13		.14		.12

Individual Animal pH Responses to Second Carbohydrate Challenge
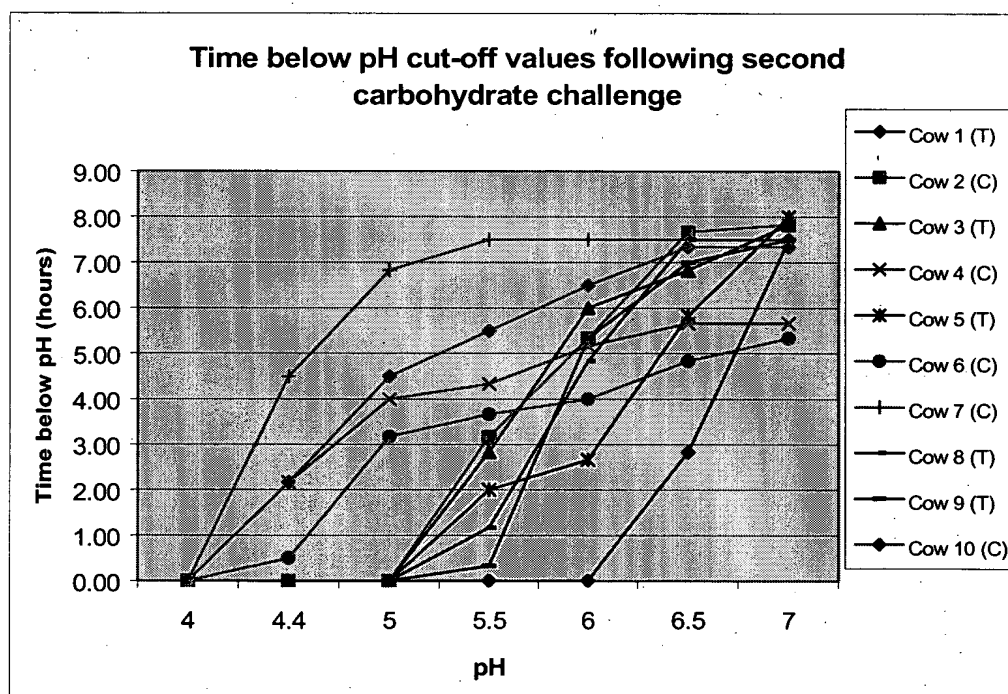
DISCUSSION/CONCLUSIONS: Twice a day acarbose treatment reduced pH responses to a high carbohydrate load and blocked L-lactate production in response to the load. The pH responses to the first challenge were similar for the two groups; it was the second challenge that allowed distinction. This is similar to the observations of Cowe, et al. (J. Anim. Sci. 77:2259, 1999) in which acute acidosis is induced after multiple challenges.